University of Alberta

MODIFICATIONS TO RNA POLYMERASE II AND TRANSCRIPTION REGULATION IN HERPES SIMPLEX VIRUS TYPE 1 INFECTED CELLS

by

Melissa Catherine Long ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of the Doctor of Philosophy

Department of Biochemistry

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With much love

To my parents

Stanley and Gwen Long

ABSTRACT

During lytic infection, herpes simplex virus type 1 (HSV-1) subverts the host cell's RNAP II transcription apparatus to efficiently express its own genes, while suppressing the expression of most cellular genes. The mechanism by which RNAP polymerase II (RNAP II) is directed to viral delayed-early (DE) and late (L) genes is unresolved. We find that following HSV-1 infection, components of the RNAP II transcription apparatus are altered. RNAP II is recruited into viral DNA replication compartments and is aberrantly phosphorylated, most likely on the large subunit carboxy-terminal domain (CTD). Furthermore, TFIIB and MO15 (TFIIH) protein levels decline, and TBP, p56 (TFIIE), RAP74 (TFIIF), MO15 (TFIIH), and MAT1 (TFIIH) are recruited into viral DNA replication compartments. These virus induced modifications to the RNAP II transcription machinery may be instrumental in achieving selective transcription of the viral genome.

The viral immediate-early (IE) protein ICP22 and the virion protein kinase UL13 are necessary for efficient modifications to RNAP II following infection. Furthermore, ICP22 and UL13 are required for normal viral gene transcription in some cell lines. We find that ICP22 coimmunoprecipitates with the viral IE transactivator ICP4, providing a further link between ICP22 and transcription activation. The ICP22 and UL13 proteins may directly or indirectly contribute to the aberrant phosphorylation of the RNAP II large subunit that follows HSV-1 infection.

In our search for CTD kinases affected by HSV-1 infection, we discovered that virus infection leads to attenuation of DNA-dependent protein kinase (DNA-PK) activity and its catalytic subunit. The loss of p350/DNA-PKcs protein and DNA-PK activity is dependent on ICP0 but may not be responsible for the aberrant phosphorylation of RNAP II after infection.

We have examined whether RNAP II modifications occur after infection with other human DNA viruses. We find that RNAP II modifications occur following infection with other members of the α-herpes family of viruses, but not after infection with β-herpesviruses or vaccinia virus.

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LIST OF ABBREVIATIONS

AS	antisense
BHK	baby hamster kidney cells
bp	base pairs
BSA	bovine serum albumin
CAK	cyclin-dependent kinase activating kinase
CAT	chloramphenicol acetyltransferase
CDK	cyclin-dependent kinase
cDNAs	complementary deoxyribonucleic acids
CIAP	calf intestinal alkaline phosphatase
CTD	carboxy-terminal domain
cpe	cytopathic effect
CV-1	African green monkey kidney
DARF	dichlorotriazinyl amino fluorescein donkey anti-rabbit
DE	delayed-early
D-MEM	Dulbecco modified Eagle's medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNA-PK	DNA-dependent protein kinase
DRB	5, 6-dichlorobenzimidazole riboside
DTT	dithiothreitol
E. Coli	*Escherichia coli*
EAP	Epstein-Barr virus small RNAs-associated protein
EBER	Epstein-Barr virus small RNAs
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EF-1δ	elongation factor 1δ
EGTA	ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid
EHV	equine herpesvirus
EIAV	equine infectious anemia virus
EICP4	equine herpesvirus homologue of HSV-1 ICP4
EICP22	equine herpesvirus homologue of HSV-1 ICP22
F-ICP22	FLAG ICP22

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAMR	rhodamine-conjugated goat anti-mouse
gC	glycoprotein C
GTF	general transcription factor
HAUSP	herpesvirus-associated ubiquitin-specific protease
HCF	host cell factor
HCMV	human cytomegalovirus
HEL	human embryonic lung cells
HeLa	human epithelioid cervical carcinoma cells
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HCl	hydrochloric acid
HHV-1	human herpes virus type 6
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
HSB-2	T lymphoblastoid cells
HSF1	heat shock factor 1
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
ICP	infected cell protein
IE	immediate-early
IgG	immunoglobulin G
Inr	initiator
L	late
LAT	latency-associated transcript
MEL	murine erythroleukemia cells
MeWos	human melanoma cells
MMTV	mouse mammary tumor virus
MOI	multiplicity of infection
MOPS	3-(N-morpholino) propanesulfonic acid
MPF	maturation-promoting factor
mRNA	messenger ribonucleic acid
mRNase	messenger ribonuclease
MW	molecular weight
ND	nuclear domain
NER	nucleotide excision repair
NPG	N-propyl gallate

OCT-1	octamer transcription factor
ORF	open reading frame
p350/DNA-PKcs	DNA-dependent protein kinase catalytic subunit formally known as p350
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline containing 0.1% Tween 20
PCV	packed cell volume
PFU	plaque forming unit
PKC	protein kinase C
PIC	preinitiation complex
PMSF	phenylmethylsulfonyl fluoride
pol	polymerase
poly (A)	polyadenylic acid
P-TEFb	positive transcription elongation factor
RNA	ribonucleic acid
RNAP II	RNA polymerase II
RNase	ribonuclease
RR	ribonucleotide reductase
rRNA	ribosomal ribonucleic acid
RSB	reticulocyte saline buffer
S	sense
SCID	severe combined immunodeficient
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
snRNA	small nuclear ribonucleic acid
snSNP	small nuclear ribonucleoprotein particles
SRB	suppressor of RNAP B (II)
TAFs	TBP-associated factors
TAK	Tat-associated kinase
TBP	TATA-box binding protein
TBS	Tris-buffered saline
TEMED	N,N,N,N′,N′-tetramethylethylenediamine
tet	tetracycline
TIF	*trans*-inducing factor
TLCK	N-α-*p*-tosyl-L-lysine chloromethyl ketone
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone

tRNA	transfer ribonucleic acid
UV	ultraviolet
v/v	volume/volume
Vero	African green monkey kidney cells
vhs	virion host shutoff
VV	vaccinia virus
VZV	varicella-zoster virus
WO	cells were washed and incubated in drug-free medium for 2 hr
WT	wild-type
w/v	weight/volume

OVERVIEW

Most of the processes that interest biologists - such as growth, differentiation, and development - are the end points of coordinated gene regulation. In order for cells to grow, differentiate and respond appropriately to internal and external signals, they must express specific subsets of genes in a precise temporal and spatial manner. Because the primary mechanism for regulating a gene's expression is to regulate its transcription, considerable research has centered on the mechanisms of eukaryotic transcription.

Viruses that replicate in the host cell nucleus and use the host's transcription machinery have been widely utilized as model systems to study eukaryotic transcription. DNA viruses such as adenovirus and herpesvirus, with their easily manipulated genomes, allow molecular biologists to employ genetic analyses of transcription regulatory mechanisms in intact mammalian cells. Some of the greatest insights into the mechanisms of eukaryotic transcription have occurred through the study of viral regulatory proteins such as E1A and VP16.

In this study, we have addressed the question of how RNA polymerase II (RNAP II) transcription is activated on some genes and repressed on others, by analyzing the changes that occur to the host transcription machinery following infection with herpes simplex virus type 1 (HSV-1). Results from this study suggest that virus-induced modifications to components of the host's RNAP II transcription apparatus may shift transcription from cellular to viral genomes in a promoter-independent fashion.

CHAPTER ONE. INTRODUCTION

A. RNA polymerase II (RNAP II)

There are three nuclear RNA polymerases (RNAPs), each responsible for transcribing a specific subset of genes: RNAP I transcribes the large ribosomal RNA (rRNA) genes, RNAP II transcribes all protein-coding and most of the small nuclear RNA (snRNA) genes, and RNAP III transcribes small nontranslated RNA genes, such as 5S rRNA, U6 snRNA and transfer RNA (tRNA) (Zawel and Reinberg, 1993; Zawel and Reinberg, 1995).

RNAP II is the central component in the transcription of class II genes. The protein is a large, multisubunit enzyme (approximately 550 kDa) containing at least 10 subunits, ranging from 10 to 240 kDa (Sawadago and Sentenac, 1990; Woychick and Young, 1990; Young, 1991; Dahmus, 1994). RNAP II activity has not been reconstituted from purified subunits. It is unknown whether all ten polypeptides are specific subunits of RNAP II or merely associated factors (Zawel and Reinberg, 1993).

The largest subunit of RNAP II appears to play an active role in transcription initiation and elongation. The RNAP II large subunit is responsible for RNA catalysis and has RNA and DNA binding functions. In support of the large subunit's role in transcription elongation, yeast and *Drosophila* RNAP II mutants which are defective in RNAP II transcription elongation *in vitro* contain mutants in the large subunit (reviewed in Sawadogo and Sentenac, 1990; Zawel and Reinberg, 1995). In addition, sensitivity to α-amanitin, an inhibitor of transcription elongation, resides in the large subunit of RNAP II (Greenleaf, 1983).

1. Carboxy-terminal domain (CTD)

The RNAP II large subunit contains a unique carboxy-terminal domain (CTD), a feature which is not shared with the large subunits of prokaryotic RNAP, eukaryotic RNAP I or III. In man and mouse, the CTD consists of 52 heptapeptide repeats of the consensus sequence YSPTSPS (Corden et al., 1985; Allison et al., 1985; Dahmus, 1994; Dahmus, 1996) and is highly conserved in eukaryotes. The CTD consensus sequence is repeated 45 and 26 to 27 times in *Drosophila* and yeast respectively (Young, 1991). The CTD is essential for cell viability. Deletion of more than half of the heptapeptide repeats in mouse, *Drosophila*, and yeast is lethal (Zawel and Reinberg, 1993; Zawel and Reinberg, 1995).

The large subunit of RNAP II is extensively modified and is the primary site of phosphorylation in RNAP II. Phosphorylation of the large subunit is thought to be confined to the CTD (Cadena and Dahmus, 1987; Baskaran et al., 1993; Dahmus, 1996) and occurs on serine, threonine and tyrosine residues (Corden, 1990; Corden and Ingles, 1992; Dahmus, 1994; Dahmus, 1996). However, serine is the predominant site of phosphorylation (Buhler et al., 1976; Cadena and Dahmus, 1987; Zhang and Corden, 1991a; Kang and Dahmus, 1995). The CTD is also modified by glycosylation by the addition of N-acetylglucosamines to serine and threonine residues (Kelly et al., 1993; Kang and Dahmus, 1995). Phosphorylation and glycosylation occur at multiple sites on the CTD and may be mutually exclusive (Dahmus, 1994).

RNAP II exists in two forms *in vivo*, IIO and IIA, which differ in the extent of CTD phosphorylation (Kim and Dahmus, 1986; Cadena and Dahmus, 1987; Dahmus, 1996). The large subunit of RNAP IIA (IIa) is nonphosphorylated and migrates at approximately 215 kDa, whereas the large subunit of RNAP IIO (IIo) is phosphorylated at up to 50 sites and migrates at approximately 240 kDa (Dahmus, 1994; Kang and Dahmus, 1995; Dahmus, 1996). RNAP IIA is thought to be involved in transcription initiation by binding to the promoter and entering into the preinitiation complex (PIC) (Payne et al., 1989; Lu et al., 1991; Dahmus, 1996). IIA is the form found in RNAP II holoenzymes (Ossipow et al., 1995). RNAP IIO is thought to be active in transcription elongation (Payne et al., 1989; Dahmus, 1996). The transition between IIA and IIO occurs early in the transcription cycle, concurrent with the transition between initiation and elongation (O'Brien et al., 1994; Dahmus, 1994; Dahmus, 1996). After completion of a round of transcription, IIO must be converted back to IIA before reinitiation of transcription can occur (Payne et al., 1989; Laybourn and Dahmus, 1990; Chesnut et al., 1992; Dahmus and Dynan, 1992; Dahmus, 1994; Dahmus, 1996).

Other forms of RNAP II have been observed *in vitro* but are likely not to exist *in vivo*. One form of the RNAP II large subunit (IIb) is approximately 180 kDa and is a derivative of IIa. The IIb subunit lacks the CTD and is the result of proteolysis during enzyme purification (Dezelee et al., 1976; Greenleaf et al., 1976; Kang and Dahmus, 1995). Intermediately phosphorylated forms between IIa and IIo can be observed during phosphorylation or dephosphorylation of the protein *in vitro* (Kim and Dahmus, 1986; Cadena and Dahmus, 1987; Zhang and Corden, 1991b). However, these intermediate forms are usually observed only in the presence of limiting concentrations of ATP (Dahmus, 1994). In addition, some intermediate forms of RNAP II are observed after heat-shock treatment (Dubois et al., 1991).

The relationship between the stoichiometry of phosphorylation and the mobility shift of the large subunit of RNAP II is complex. The mobility shift of the RNAP II large subunit is not linear with respect to the number of phosphates incorporated. The addition of approximately 40 phosphates to native calf thymus IIa is sufficient to shift the protein to the IIo position on SDS-PAGE gels. Furthermore, phosphorylation of the native IIo subunit at an additional 10 to 20 sites does not cause further mobility shifts in the protein, indicating that the maximum mobility shift is observed when only a fraction of the sites have been phosphorylated (Payne and Dahmus, 1993). Phosphorylation of the recombinant mouse CTD gives slightly different results. The addition of 7 to 9 phosphates to recombinant mouse CTD, containing 54 heptapeptide repeats, is sufficient for the maximal shift in mobility from IIa to IIo (Zhang and Corden, 1991b).

To date, the stoichiometry of RNAP II large subunit phosphorylation *in vivo* has not been determined. *In vitro* phosphorylations show that 0.5 to 1 phosphate per repeat results in a mobility shift comparable to that of phosphorylated IIo, suggesting that a minimum of 25 to 50 phosphorylation sites are present in IIo (Payne and Dahmus, 1993; Dahmus, 1994).

The ratio of RNAP IIA and RNAP IIO abundance varies in different cell types. It has been reported that RNAP IIO is the predominant form in exponentially growing HeLa S3 cells, whereas RNAP IIA is the prevalent form in bovine kidney cells and calf thymus (Dahmus, 1994). This ratio is dramatically altered in response to different conditions such as heat shock (Dubois et al., 1994a), serum induction (Dubois et al., 1994b), and poliovirus infection (Rangel et al., 1987). Poliovirus infected HeLa S3 cells contain significant decreases in total and chromatin bound RNAP IIO. The loss of RNAP IIO requires the expression of the poliovirus genome (Rangel et al., 1987; Rangel et al., 1988). After heat shock, HeLa S3 cells contain an increased level of hyperphosphorylated RNAP II. In serum-stimulated cells, the abundance of RNAP IIO increases (Dubois et al., 1994b).

The CTD is believed to play a critical role in transcription *in vivo*. One of proposed functions of the CTD is that it interacts with members of the basal and activated transcription complex, such as TFIID (Koleske et al., 1992; Usheva et al., 1992) and GAL4 (Allison and Ingles, 1989; Scafe et al., 1990; Liao et al., 1991), thereby recruiting and orienting RNAP II at the promoter. The CTD may also be involved in initiation by directly interacting with DNA (Suzuki, 1990). Many of these interactions may help stabilize the holoenzyme at the promoter.

The CTD may play an indirect role in chromatin remodeling. The CTD associates with the SRB/SWI/SNF mediator, a complex which plays a role in transcription initiation *in vivo* and is an integral member of the RNAP II holoenzyme (Koleske and Young, 1995). The SRB/SWI/SNF complexes of yeast holoenzymes remodel chromatin and prevent nucleosome mediated repression. In support of this, in yeast, both SWI and CTD mutations are complemented by mutations in an HMG protein (SIN1) and histone H3 (SIN2) respectively (Peterson and Herskowitz, 1992). Alterations in chromatin structure may facilitate PIC assembly on promoters. The mammalian homologue of the SWI/SNF complex disrupts nucleosomes and facilitates the binding of TBP and GAL4-VP16 to DNA (Imbalzano et al., 1994; Kwon et al., 1994; Peterson and Tamkun, 1995; Wilson et al., 1996; Gaudreau et al., 1997).

The CTD also appears to be important in regulated transcription. A deletion in the CTD is defective in its response to activator proteins (Allison and Ingles, 1989; Liao et al., 1991; Gerber et al., 1995). Because of deletions in the CTD, there may be a loss in SRB/SWI/SNF association. Deletion of the SRB2 and SRB5 genes diminish transcription activation in yeast (Thompson et al., 1993). The SRB/SWI/SNF complex appears to mediate the holoenzyme's response to activators (Kim et al., 1994; Liao et al., 1995; Wilson et al., 1996).

Once a PIC is assembled at the promoter, a CTD kinase may phosphorylate the CTD, releasing the CTD from its contacts with the SRB/SWI/SNF complex and other GTFs. RNAP II may then be allowed to elongate (Koleske and Young, 1995). The phosphorylated CTD, which is highly negatively charged, may then act as a cowcatcher, dispelling histones and other inhibitory DNA binding proteins from the template during transcription elongation (Corden, 1990; Peterson et al., 1991; Corden and Ingles, 1992).

Recently it has been suggested that the CTD plays a role in nucleotide excision repair (NER), a process where a single-strand of damaged DNA is removed and new DNA is synthesized (Svejstrup et al., 1996). As TFIIH contains multiple subunits that are involved in NER, this raises the possibility that the CTD may function in transcription coupled DNA repair (Kang and Dahmus, 1995). Evidence exists in the literature to support the hypothesis that transcription and DNA repair are coupled (Drapkin et al., 1994b). DNA damage in transcribed genes is repaired at a much faster rate than in nontranscribed genes. Furthermore, repair occurs preferentially to the transcribed strand of DNA (Drapkin et al., 1994b; Orphanides et al., 1996). Sites of DNA damage may cause RNAP II stalling. It is possible that during this stalling process, RNAP IIO becomes dephosphorylated to RNAP IIA. The presence of RNAP IIA at the DNA lesion may recruit TFIIE and TFIIF, GTFs which interact with the unphosphorylated CTD. TFIIE may then recruit TFIIH to the site of damage and TFIIH may then aid in the further recruitment of additional repair proteins. The CTD kinase activity of TFIIH may then convert IIA to IIO, resulting in the elongation competent form of RNAP II (Kang and Dahmus, 1995; Dahmus, 1996).

Another proposed CTD function includes the recruitment of splicing factors. Proteins involved in splicing, including a subset of small nuclear ribonucleoprotein particles (snRNPs) and serine/arginine-rich SR splicing factors, interact with RNAP II (Mortillaro et al., 1996; Yuryev et al., 1996; Du and Warren, 1997). Interaction with splicing components is localized to the CTD of the RNAP II large subunit (Yuryev et al., 1996; Du and Warren, 1997). Mortillaro et al. (1996) and Kim et al.'s (1997a) experiments indicate that splicing components interact with the hyperphosphorylated form of RNAP II. RNAP II colocalizes with splicing components (snRNPs and non-snRNP splicing factors) in the nucleus, suggesting that the protein is associated with pre-mRNA processing *in vivo* (Bregman et al., 1995; Mortillaro et al., 1996). Certain cleavage and polyadenylation factors also interact with the CTD. Furthermore, splicing, 3′ end processing and termination appear to be defective in the presence of a CTD mutant (McCracken et al., 1997). These results suggest that the CTD may play a direct role in pre-mRNA processing.

It is possible that transcription and pre-mRNA processing are linked functionally. McCracken et al. (1997) propose that an mRNA factory exists to carry out transcription, splicing, and cleavage-polyadenylation of mRNA. Nascent transcripts may mature within these mRNA factories, which may also be present at the promoter. Polyadenylation factors have been copurified with a RNAP II holoenzyme that is competent for transcription initiation (McCracken et al., 1997).

2. CTD protein kinases

CTD protein kinases are defined as enzymes that phosphorylate the consensus heptapeptide YSPTSPS repeat and cause the characteristic electrophoretic mobility shift in IIa or in recombinant CTD (Dahmus, 1994). Many CTD kinases have been characterized *in vitro* but the physiological significance of these CTD kinases *in vivo* remains to be determined. Because the CTD can be phosphorylated on serine, threonine and tyrosine residues, it seems likely that more than one CTD kinase may be involved in CTD phosphorylation.

It is possible that different CTD kinases function at different stages in transcription or the cell cycle. In addition, different CTD kinases may function when the cells are in different physiological states. For instance, phosphorylation of the CTD may facilitate promoter clearance of RNAP II, but earlier phosphorylation of the CTD during PIC assembly may lead to the premature release of RNAP II from promoters (Dahmus, 1994). There is evidence to support this hypothesis. Phosphorylation of the CTD by the $p34^{cdc2}$ gene product, described in further detail later in this chapter, results in the disruption of DAB/RNAP II/F complexes (Zawel et al., 1993). The dissociation of RNAP II from the PIC may result in a down-regulation of transcription. Furthermore, if a CTD kinase preferentially phosphorylates free RNAP II, a decreased amount of RNAP IIA would be available for PIC formation. It is currently unknown as to whether discrete CTD kinases act at different steps in transcription.

There have been a variety of CTD kinases identified in species ranging from mammals to yeast (Dahmus, 1994). I will further describe the known mammalian CTD kinases.

a. TFIIH

CTD kinase activity is associated with the basal transcription factor TFIIH and is a component of the RNAP II holoenzyme (Ossipow et al., 1995). The subcomplex consisting of three subunits of TFIIH (MAT1, cyclin H, and CDK7/MO15) possesses CTD kinase activity *in vitro* (Svejstrup et al., 1996). The preferred substrate of TFIIH is RNAP II that is assembled into PICs. TFIIH phosphorylation on the CTD of RNAP II in a PIC is stimulated by the presence of recombinant TBP and TFIIB (Lu et al., 1992). TFIIE also stimulates TFIIH's phosphorylation of the CTD (Orphanides et al., 1996).

TFIIH is required for *in vitro* transcription catalyzed by RNAP IIB, which lacks the CTD (Zawel and Reinberg, 1993). Therefore, TFIIH may perform dual functions in transcription by not only phosphorylating the CTD but also catalyzing a step in transcription that is independent of the CTD (Dahmus, 1994). TFIIH is described in more detail in this chapter in section C.1.a)

b. CDK8 and cyclin C

Cyclin-dependent kinases (CDKs) are controlled by association with cyclin partners and many CDKs regulate cell growth by phosphorylating key substrates. These phosphorylation events may either be activating or inhibitory phosphorylations (reviewed in Clarke, 1995). Human CDK8 (also called K35), the kinase partner of cyclin C, is a component of the

RNAP II holoenzyme (Chao et al., 1996). CDK8 and cyclin C are the human homologues of yeast SRB10 and SRB11 proteins (Tassan et al., 1995). Yeast holoenzymes lacking SRB10 and SRB11 are deficient in CTD phosphorylation *in vivo* (Liao et al., 1995). Furthermore, CDK8 and cyclin C complexes are capable of phosphorylating recombinant CTD *in vitro* (Rickert et al., 1996). Although no experiments have been performed to determine the CTD residues phosphorylated by CDK8/cyclin C, CDKs are known to be serine/threonine kinases (Clarke, 1995).

It remains to be seen whether the phosphorylation of RNAP II by CDK8/cyclin C is related to cell cycle control. Cyclin/CDK complexes may not function exclusively in cell cycle regulation, but may be implicated in other cellular processes. For instance, the yeast PHO80-PHO85 cyclin-CDK complex phosphorylates PHO4, a transcriptional activator of the Pho5 gene. As a result, PHO4 becomes inactive and the Pho5 gene is down-regulated. This particular repression is important in a pathway that senses inorganic phosphate in yeast and appears not to be related to cell cycle control (Kaffman et al., 1994).

Further studies show that the adenovirus E1A and the HSV-1 viral transactivator VP16 interact with CDK8 and that these interactions are dependent on the activation domains of these activators (Herrmann et al., 1996). This suggests that the interactions between these activators and CDK8 are relevant for VP16 and E1A transactivation functions. Also, further experiments imply that VP16 and E1A associate with the RNAP II holoenzyme (Gold et al., 1996).

c. $p34^{cdc2}$

The $p34^{cdc2}$ gene product is a protein that is involved in cell cycle regulation, is a component of the maturation promoting factor (MPF), and is an mitosis specific histone H1 kinase (Nurse, 1990). The CTD has also been found to be phosphorylated by the mammalian homologue of *Schizosaccharomyces pombe* $p34^{cdc2}$, suggesting that the CTD may be involved in cell cycle regulation of transcription (Cisek and Corden, 1989).

CTD kinase activity is dependent on $p34^{cdc2}$ association with mouse cyclin B or a 58 kDa subunit (Cisek and Corden, 1991). A complex of $p34^{cdc2}$

and the 58 kDa subunit, designated as E2, phosphorylates serine 2 and threonine 5 in the consensus repeat. There are 15 to 20 phosphates incorporated among the 52 heptapeptide repeats by the E2 kinase. Moreover, the ratio of phosphoserine and phosphothreonine observed during *in vitro* phosphorylation of the CTD by E2 is similar to that observed during *in vivo* phosphorylation of the CTD (Zhang and Corden, 1991a).

As described earlier in this chapter, the phosphorylation of the CTD by MPF disrupts DAB/RNAP II/F complexes. When the fully phosphorylated form of RNAP II, isolated from human cells, forms the initial DAB/RNAP II/F complex, MPF treatment results in the disruption of the complex. This suggests that MPF phosphorylates the CTD at sites other than those recognized by other cellular kinases or the extent of MPF phosphorylation is greater than that which occurs *in vivo*. The ability of MPF to disrupt PICs is interesting especially since MPF activity is at a maximum during mitosis when RNAP II transcription is dramatically reduced. MPF may be involved in mitotic transcriptional repression by phosphorylating the CTD and disrupting PICs (Zawel et al., 1993).

d. CTD-K1 and CTD-K2

CTD-K1 and CTD-K2 are two CTD kinases that have been purified from HeLa transcription extracts (Payne and Dahmus, 1993). These kinases differ in their phosphorylation of the CTD and in their substrate specificity. CTD-K1 incorporates 33 moles phosphate per mole of IIa at a ratio of 30 phosphoserines to 1 phosphothreonine. CTD-K2 incorporates 50 moles phosphate per mole of IIa at a ratio of 9 phosphoserines to 1 phosphothreonine (Dahmus, 1994).

e. Tat-associated kinase (TAK)

Tat-associated kinase (TAK) phosphorylates serines and threonines on the CTD of the large subunit of RNAP II *in vitro* (Herrmann and Rice, 1993; Herrmann and Rice, 1994). TAK also phosphorylates a 42 kDa (Herrmann and Rice, 1993) protein which may be a component of TAK

(Yang et al., 1996). The TAK kinase associates with the transactivator Tat proteins of human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) and the distantly related lentivirus equine infectious anemia virus (EIAV). The Tat protein regulates viral gene expression and is necessary for efficient viral replication. Tat binds to an HIV RNA element known as TAR, located at the 5' ends of nascent viral transcripts. Tat consists of two domains, one of which is the RNA binding domain and the other which is the activation domain. Tat appears to stimulate both transcription initiation and elongation. Binding of Tat to TAR may enable the Tat activation domain to interact with the RNAP transcription machinery in order to recruit cellular transcription factors to viral promoters (reviewed in Jones and Peterlin, 1994). Tat protein also interacts with TBP or the TFIID complex *in vitro* (Kashanchi et al., 1994). The CTD is required for Tat function *in vivo*. Tat may stimulate transcription elongation by recruiting TAK and enhancing CTD phosphorylation (Yang et al., 1996).

TAK is a novel kinase activity, distinct from previously described CTD kinases such as TFIIH (Svejstrup et al., 1996), $p34^{cdc2}$ (Cisek and Corden, 1989), CTD-K1 and CTD-K2 (Payne and Dahmus, 1993), c-Abl (Kipreos and Wang, 1992; Baskaran et al., 1993; Wang, 1993), and DNA-PK (Dvir et al., 1992; Anderson, 1994). TFIIH is not detected in TAK complexes. TAK is unlike the $p34^{cdc2}$ kinase since TAK does not phosphorylate histone H1. CTD-K1 and CTD-K2 do not associate with Tat proteins. Furthermore, DNA is not present in TAK assays, a requirement that is necessary for DNA-PK (Herrmann and Rice, 1994) and c-Abl (Kipreos and Wang, 1992; Baskaran et al., 1993; Wang, 1993) activity.

f. c-Abl

c-Abl is a nuclear CTD kinase that binds directly to DNA (Kipreos and Wang, 1992; Wang, 1993). c-Abl phosphorylates the CTD on tyrosine residues at approximately 30 sites (Baskaran et al., 1993) All other reported CTD kinases phosphorylate serine/threonine residues. It is important to note that c-Abl is not the only tyrosine kinase that phosphorylates the CTD because a c-Abl null mutant cell line is also capable of phosphorylating the CTD on tyrosine residues. In addition, there are comparable levels of tyrosine phosphorylation on IIo in interphase and metaphase HeLa cells, suggesting that tyrosine phosphorylation of the CTD is not cell-cycle dependent (Dahmus, 1994).

g. DNA-dependent protein kinase (DNA-PK)

DNA dependent protein kinase (DNA-PK) is a putative CTD kinase, which is composed of a catalytic subunit (DNA-PK catalytic subunit (DNA-PKcs), formally known as p350) (Carter et al., 1990; Lees-Miller et al., 1990; Hartley et al., 1995) of approximately 460 kDa and a dimeric protein of 70 and 80 kDa, known as Ku (Anderson, 1994).

DNA-PK was originally identified as a CTD kinase from a system using an immobilized DNA template that allows the isolation of PICs (Arias and Dynan, 1989). A kinase capable of phosphorylating endogenous, promoter-bound RNAP II was present in the assembled PICs (Laybourn and Dahmus, 1990; Arias et al., 1991). Subsequent experiments recovered DNA-PK from the assembled PICs (Dvir et al., 1992; Peterson and Herskowitz, 1992). Eluted DNA-PK from the PICs efficiently phosphorylated a CTD substrate fused to the DNA-binding domain of GAL4. GAL4-CTD phosphorylation is dependent on the presence of DNA and a DNA-binding domain in the substrate (Peterson and Herskowitz, 1992). DNA-PK purified from PICs formed on immobilized DNA templates consists of both p350/DNA-PKcs and Ku (Dvir et al., 1992).

Ku binds to free ends of double stranded DNA without any apparent sequence specificity (Gottlieb and Jackson, 1994) and is the DNA binding component of DNA-PK, targeting DNA-PK to DNA (Dvir et al., 1992; Gottlieb and Jackson, 1993). *In vitro*, Ku binds to DNA ends and then translocates along the double helix in an ATP-independent manner (Paillard and Strauss, 1991; Griffith et al., 1992). Ku also binds to DNA containing nicks and gaps and to DNA containing single and double strand transitions (Blier et al., 1993, Falzon et al., 1993). In addition to its DNA targeting functions, Ku protects DNA ends from exonucleolytic degradation (Anderson, 1994) and may also act as a helicase (Tuteja et al., 1994).

i. DNA-PK activity

DNA-PK is a serine/threonine kinase that requires free ends of double-stranded DNA for activation (Carter et al., 1990; Anderson and Lees-Miller, 1992; Gottlieb and Jackson, 1993; Morozov et al., 1994). The consensus sequence for DNA-PK phosphorylation is a serine or threonine directly followed by a glutamine. Binding of the DNA-PK substrate to DNA increases substrate phosphorylation efficiency and may also be a determinant for substrate recognition by DNA-PK (reviewed in Anderson, 1994).

The substrates of DNA-PK include p53, serum response factor, c-Fos, c-Jun, octamer transcription factor (OCT-1), the simian virus 40 large T antigen, Sp1, TFIID, and the CTD of the large subunit of RNAP II. These substrates are only phosphorylated efficiently when they are bound to the same DNA molecule as DNA-PK, suggesting that phosphorylation by DNA-PK occurs close to the site of DNA-PK activation (Lees-Miller et al., 1990; Anderson and Lees-Miller, 1992; Anderson, 1994; Anderson et al., 1995; Jeggo et al., 1995).

ii. DNA-PK functions

DNA-PK functions in DNA double-strand break repair and V(D)J recombination (Anderson, 1994; Jeggo et al., 1995; Roth et al., 1995). Severe combined immunodeficient (SCID) mice are defective in DNA repair and V(D)J recombination and lack the p350/DNA-PKcs subunit of DNA-PK and DNA-PK activity. Repair and recombination activities are restored when SCID cells are complemented with chromosome fragments expressing p350/DNA-PKcs (Blunt et al., 1995; Kirchgessner et al., 1995; Peterson et al., 1995). Furthermore, murine cell lines which are radiation sensitive and V(D)J recombination-deficient lack the p80 Ku subunit of DNA-PK and are defective in DNA end binding activity. When extracts are supplemented with Ku or when Ku defective cells are transfected with p80 Ku, repair and recombination activities are restored (Getts and Stamato, 1994; Rathmell and Chu, 1994a; Rathmell and Chu, 1994b; Smider et al., 1994; Taccioli et al., 1994; Boubnov et al., 1995; Finnie et al., 1995). A radiosensitive human cell line which is defective in DNA double-strand break repair lacks p350/DNA-PKcs and DNA-PK activity (Lees-Miller et al., 1995).

There are reports to suggest that DNA-PK is involved in transcription. DNA-PK is responsible for the *in vitro* phosphorylation of many transcription factors and DNA binding proteins (Lees-Miller et al., 1990; Anderson and Lees-Miller, 1992; Anderson, 1994; Anderson et al., 1995; Jeggo et al., 1995), and is found in the RNAP II holoenzyme (Maldonado et al., 1996). DNA-PK phosphorylates the CTD on serine and threonine residues with similar efficiencies, a result that is different from that observed with other CTD kinases (Payne et al., 1989; Zhang and Corden, 1991a; Lu et al., 1992). This suggests that the recognition sites within the CTD are different between DNA-PK and other reported CTD kinases. Furthermore, the CTD does not contain the normal consensus sequence recognized by DNA-PK (Anderson, 1994). Glutamine does not follow serine or threonine in the CTD, suggesting that DNA-PK phosphorylates alternate consensus sequences (Anderson, 1994).

DNA-PK may regulate RNAP I and RNAP II transcription through its phosphorylation abilities (Kuhn et al., 1995; Labhart, 1995; Giffin et al., 1996; Giffin et al., 1997). DNA-PK may repress RNAP I transcription initiation, but not transcription elongation, by phosphorylating a component of the RNAP I transcription initiation complex (Kuhn et al., 1995; Labhart, 1995). In addition, DNA-PK repression of RNAP II transcription has been observed in glucocorticoid induced transcription from the mouse mammary tumor virus promoter. DNA-PK induced phosphorylation of the glucocorticoid receptor and OCT-1 may cause repression (Giffin et al., 1996; Giffin et al., 1997).

In support of DNA-PK's role in class II gene transcription, DNA-PK activity is stimulated by some RNAP II transcription activators *in vitro* (Peterson et al., 1995). The human heat shock factor 1 (HSF1) and a transcriptionally active derivative of GAL4 stimulate DNA-PK. This kinase activation is dependent on the presence of DNA containing activator binding sequences. In addition, both DNA binding and activation domains present in the activator proteins are required for DNA-PK stimulation. These results suggest that the stimulation of DNA-PK by these activator proteins is dependent on their binding to the DNA template. It is likely that DNA-PK is bound to the DNA when DNA-PK activation occurs. The stimulation of DNA-PK activity by activators may facilitate the phosphorylation of various transcription regulatory proteins, which could lead to either activation or repression of transcription (Peterson et al., 1995).

h. Positive transcription elongation factor (P-TEFb)

The positive elongation factor (P-TEFb), consisting of two subunits (43 and 124 kDa) (Marshall and Price, 1995), is capable of phosphorylating native RNAP II on its CTD, most likely on serine and threonine residues (Marshall et al., 1996). P-TEFb is different from the other putative CTD kinases in that it is a factor that prevents elongation arrest, by helping RNAP II pass through nucleoprotein complexes and DNA sequences that act as intrinsic arrest sites (Marshall et al., 1996; Reines et al., 1996). The other CTD kinases described above are thought to act during the transition between initiation and elongation, whereas P-TEFb phosphorylates RNAP II during elongation (Marshall et al., 1996). P-TEFb is distinct from other putative CTD kinases in its subunit composition and functional properties. TFIIH does not functionally substitute for P-TEFb, and appears to be unrelated to P-TEFb (Marshall et al., 1996).

P-TEFb is not associated with PIC complexes (Marshall and Price, 1992; Marshall and Price, 1995), but phosphorylates RNAP II when the polymerase is present in an early elongation complex (Marshall et al., 1996). Fully phosphorylated RNAP II may be required throughout the duration of transcription elongation. P-TEFb may be responsible for phosphorylating RNAP II that has been fully or partially dephosphorylated at intrinsic pause sites. When unphosphorylated or incompletely phosphorylated forms of RNAP II are converted back to IIO, transcription elongation may continue.

3. CTD phosphatases

Little is known about the activity or structure of phosphatases that dephosphorylate RNAP IIO to RNAP IIA. Transcription elongation is carried out by RNAP IIO, and the CTD must be dephosphorylated after completion of the transcription cycle in order for reinitiation to occur. One CTD phosphatase of approximately 200 kDa, purified from HeLa transcription extracts, is capable of dephosphorylating RNAP IIO *in vitro* (Chambers and Dahmus, 1994). The CTD phosphatase is a type 2C phosphatase, requires magnesium ions for activity, and has substrate specificity for serine and threonine residues in the CTD (Chambers and Dahmus, 1994). The phosphatase does not dephosphorylate RNAP IIO that has been phosphorylated by the c-Abl tyrosine kinase *in vitro*. In addition, the CTD phosphatase interacts with RNAP II at a site distinct from the CTD (Chambers et al., 1995). Recently, a yeast CTD phosphatase similar to the HeLa S3 CTD kinase has been isolated (Chambers and Kane, 1996).

TFIIF stimulates CTD phosphatase activity, whereas TFIIB inhibits the stimulation by TFIIF. However, TFIIB has no effect on the CTD phosphatase in the absence of TFIIF. TFIIF may improve the interaction between the CTD phosphatase and RNAP II. As TFIIF is a component of the RNAP II holoenzyme prior to PIC assembly on the promoter, TFIIF may enhance the dephosphorylation of RNAP II between termination of one cycle and initiation of the next cycle. It is possible that the presence of TFIIB in the PIC counteracts the stimulatory effects of TFIIF on CTD phosphatase, allowing CTD phosphorylation to occur during promoter clearance (Chambers et al., 1995).

B. RNAP II promoters

Accurate transcription by RNAP II is dependent on the presence of specific DNA sequences, which act as signals to direct RNAP II and the general transcription factors (GTFs) to the initiation site. All protein-coding genes contain a minimal promoter, which consists of a start site (cap site) and one or both of a TATA box and pyrimidine rich initiator (Inr) element. The minimal promoter is capable of directing the formation of the transcription PIC (Zawel and Reinberg, 1993; Zawel and Reinberg, 1995; Goodrich et al., 1996). The sequences of the TATA box and Inr element and the distance between them vary among RNAP II promoters. The TATA box is located approximately 30 nucleotides upstream of the transcription initiation site, whereas the pyrimidine rich Inr encompasses the transcription initiation site (Zawel and Reinberg, 1995; Orphanides et al., 1996).

Other *cis*-acting sequences are not required for basal transcription but play a role in activated or repressed transcription. These sequences are located either a few hundred base pairs (promoter-proximal elements) or thousands of base pairs (promoter-distal elements) away from the transcription start site and are bound by DNA binding proteins, such as activators and repressors. These *cis*-acting sequences are gene-specific and are variable in number and in their order of arrangement in each gene promoter (reviewed in Zawel and Reinberg, 1993; Zawel and Reinberg, 1995).

Activators and repressors are described in further detail in section D. One type of promoter-distal element, the enhancer, is typically located thousands of nucleotides upstream or downstream of the promoter. In addition, enhancers function in both orientations (Orphanides et al., 1996; Zawel and Reinberg, 1996) and work "universally," enhancing transcription from any promoter to which they are attached (Ptashne and Gann, 1990).

C. RNAP II transcription

RNAP II transcription can be divided into five distinct stages: preinitiation complex (PIC) formation, initiation, promoter clearance, elongation, and termination. PIC formation entails the assembly of transcription proteins on a DNA template containing a promoter. Initiation involves the formation of the first RNA phosphodiester bond. Promoter clearance is the process by which RNAP II leaves the promoter and the PIC. Elongation occurs when RNAP II travels down the gene and synthesizes the nascent RNA transcript. Termination is the last stage in transcription where RNA synthesis ends and RNA is released from the DNA template. Each of these stages is a potential transcription regulatory step.

1. PIC formation

In order to initiate transcription, RNAP II requires a complex array of auxiliary factors, the GTFs. The GTFs include TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH and are conserved from yeast to humans. RNAP II and the GTFs, in the absence of other transcription factors, can initiate transcription on some promoters *in vitro*. This transcription is termed basal transcription (reviewed in Zawel and Reinberg, 1993; Zawel and Reinberg, 1995; Roeder, 1996).

There are currently two models for RNAP II PIC assembly: the multistep and holoenzyme models. The multistep model involves the stepwise addition of GTFs to the DNA. The holoenzyme model envisions the recruitment of a large preformed RNAP II complex containing many, if not all, GTFs.

a. Multistep model of PIC assembly

In the multistep model of PIC assembly, PIC formation commences with the specific binding of TFIID to the TATA box. TFIID provides a foundation for the recruitment of other transcription factors and RNAP II to the PIC through a series of protein-protein interactions. TFIID is 38 kDa and consists of the TATA box binding protein (TBP) and at least twelve TBP associated factors (TAFs), ranging from 18 to 250 kDa (Goodrich and Tjian, 1996; Verrijzer and Tjian, 1996; Tansey and Herr, 1997). The TAFs are dispensable for basal transcription but are generally required for activated transcription, suggesting that one or more of the TAFs function as coactivators (Zawel and Reinberg, 1993; Roeder, 1996; Tansey and Herr, 1997).

When TBP is bound to the TATA box, TFIIB becomes associated to produce the DB complex. TFIIA, consisting of three subunits (12, 19, and 35 kDa), also associates with TFIID but is not required for DB complex formation. Rather, TFIIA stabilizes TBP-TATA box complexes. *In vitro*, TFIIA does not appear to be essential for basal transcription from TATA containing promoters using purified GTFs. Instead, TFIIA functions as a coactivator and is required for activated transcription on certain promoters

(Roeder, 1996). In addition, TFIIA is involved in antirepression, the process by which TFIIA relieves the repressive effects of certain inhibitory proteins. Interactions between TFIIA and TBP may displace repressors which bind to TBP and prevent TFIIB recruitment into PICs. The DAB complex is required for the subsequent association of other GTFs and RNAP II in PIC formation (Zawel and Reinberg, 1995; Orphanides et al., 1996).

TFIIB also stabilizes TBP-TATA box complexes. TFIIB and TFIIA interact with TBP-TATA box complexes independently and do not directly contact each other. This is in accordance with the distinct functions of TFIIA and TFIIB (Roeder, 1996). TFIIB is thought to be the molecular bridge between TBP and TFIIF/RNAP II, forming contacts with all three proteins (Zawel and Reinberg, 1993; Zawel and Reinberg, 1995; Orphanides et al., 1996). In addition, studies in yeast suggest that TFIIB plays a role in RNAP II transcription start site selection. Mutations in the yeast TFIIB gene alter the position where RNAP II starts transcribing. It is possible that interactions between TFIIB and RNAP II affect the usage of specific transcription start sites (Orphanides et al., 1996).

The DAB complex binds the preformed TFIIF/RNAP II subcomplex. TFIIF binds to RNAP II and plays a role in delivering RNAP II to the assembling PIC on DNA. The formation of the DAB-RNAP II/F complex provides the foundation for the association of TFIIE and then TFIIH (Zawel and Reinberg, 1993; Orphanides et al., 1996).

TFIIF, in addition to targeting RNAP II to the promoter, also plays an indirect role by reducing RNAP II's binding to nonspecific DNA (Roeder, 1996). TFIIF consists of two subunits, RAP30 and RAP74 . RAP30 is able to independently recruit RNAP II to the DAB complex, but the DAB-RNAP II/RAP30 complex is less stable than the DAB-RNAP II/F complex, suggesting that RAP74 stabilizes the complex. RAP30 alone is unable to reconstitute transcription systems, indicating that both TFIIF subunits are required for transcription (reviewed in Zawel and Reinberg, 1993; Zawel and Reinberg, 1995; Orphanides et al., 1996). RAP74 stimulates transcription elongation. Furthermore, RAP74 has been implicated in transcription start site selection. Yeast mutants in TFIIB that start transcription at alternate sites are suppressed by mutations in yeast RAP74, restoring the usage of the normal transcription start site (Orphanides et al., 1996).

TFIIE association with the PIC is mediated by direct interactions with RNAP II and possible interactions with TFIIF and TBP (Roeder, 1996). TFIIE is a tetramer consisting of two p34 and two p56 kDa subunits and plays a role in promoter melting. TFIIE directly contacts TFIIH and recruits it to the DAB-RNAP II/FE complex (Maxon et al., 1994). Once bound, TFIIH stabilizes TFIIE association to the PIC (Roeder, 1996).

TFIIH is a multifunctional protein consisting of up to 9 polypeptides, ranging from approximately 30 kDa to 90 kDa (Orphanides et al., 1996; Svejstrup et al., 1996). TFIIH phosphorylates the CTD of RNAP II *in vitro*, an event that may facilitate the shift from transcription initiation to elongation (Lu et al., 1992; Serizawa et al., 1992). In addition, TFIIH possesses bi-directional helicase activity (Serizawa et al., 1993; Drapkin et al., 1994a; Roy et al., 1994), which may be required for promoter clearance (Svejstrup et al., 1996). TFIIE is thought to play a role in the regulation of TFIIH kinase and helicase activities (Drapkin and Reinberg, 1994; Ohkuma and Roeder, 1994). TFIIE stimulates TFIIH phosphorylation of RNAP II large subunit's CTD (Roeder, 1996; Svejstrup et al., 1996).

TFIIH may be involved in NER (Drapkin et al., 1994b; Schaeffer et al., 1994; Svejstrup et al., 1996). The DNA repair protein ERCC3, which is essential for NER, is the 89 kDa subunit of TFIIH. ERCC3 possesses helicase activity and may unwind the DNA helix around the site of damage in order for endonucleases to gain access during NER. Other TFIIH subunits are also DNA repair proteins. These include the helicase ERCC2, the 62 kDa subunit, the SSL1 subunit, and potentially the 34 kDa subunit (Orphanides et al., 1996). Microinjection of TFIIH into human repair deficient cells restores repair functions (Svejstrup et al., 1996). Transcription and NER may be functionally coupled events. In support of this idea, DNA damage in transcribed genes is repaired at a much faster rate than in nontranscribed genes (Orphanides et al., 1996).

In vitro, the complex containing TFIIH subunits MO15/CDK, cyclin H, and MAT1 (CDK assembly factor) functions as a CDK activating kinase (CAK) (Svejstrup et al., 1996). The CAK moiety of TFIIH also phosphorylates the CTD of the large subunit of RNAP II. The functional significance of TFIIH CAK activity *in vivo* remains to be determined. CAK's function in cell cycle control may be separate from its function in transcription. There are at least two separate cellular pools of CAK with distinct activities and only 20% of CAK is associated with TFIIH (Mäkelä et al., 1995; Shiekhattar et al., 1995; Drapkin et al., 1996). Certain CAK complexes are unable to phosphorylate the CTD of the RNAP II large subunit present in the context of PICs. These CAK complexes may lack components of TFIIH which may be required to tether TFIIH to the PIC. In addition, CAK possesses different substrate specificity when complexed with TFIIH subunits (Shiekhattar et al., 1995; Orphanides et al., 1996).

b. Holoenzyme model of PIC assembly

The term holoenzyme describes large stable complexes which contain RNAP II and associated factors. The isolation of RNAP II holoenzymes from yeast and humans has led to the formation of the holoenzyme model of PIC assembly. In this model, preassembled transcriptionally competent complexes may be recruited to promoters in one step after the binding of TBP to the TATA box (Koleske and Young, 1995).

A yeast holoenzyme containing RNAP II, TFIIF, TFIIB, TFIIH, suppressors of RNAP B (II) (SRBs), SWI, and SNF has been isolated (Koleske and Young, 1995; Wilson et al., 1996). A complex of nine SRB proteins, also known as mediator, plays a role in transcription initiation *in vivo* and is essential for normal cell growth (Koleske and Young, 1995). SWI and SNF are positive gene regulators which are involved in chromatin remodeling. SWI and SNF are thought to oppose nucleosome mediated repression by altering chromatin structure and enabling the RNAP II machinery to associate with the DNA. Both SWI and SNF are components of the mediator complex, and are associated with the CTD of the RNAP II large subunit. The presence of the SRB/SWI/SNF complex in the holoenzyme may provide the holoenzyme with the ability to disrupt nucleosomes on the DNA template during transcription initiation. This may facilitate the stable association of the PIC to the promoter (Wilson et al., 1996).

Various mammalian holoenzymes have also been isolated (Koleske and Young, 1995; Ossipow et al., 1995; Chao et al., 1996; Maldonado et al., 1996). One mammalian holoenzyme contains RNAP II, TFIIE, TFIIF, TFIIH, and the human homologues of yeast SRB7, SRB10, and SRB11 (Maldonado et al., 1996). Interestingly, SRB10 and SRB11 show homology to human CDK8 and cyclin C respectively (Tassan et al., 1995). Other proteins also copurify with mammalian holoenzymes. These include TFIID, TFIIB, YY1 (a sequence-specific protein that may be involved in Inr transcription from TATA-less promoters), DNA replication proteins, DNA-PK, and certain RNA processing factors (Ossipow et al., 1995; Chao et al., 1996; Maldonado et al., 1996; McCracken et al., 1997). Human homologues of the SWI/SNF complex are also involved in chromatin remodeling (Imbalzano et al., 1994; Kwon et al., 1994; Peterson and Tamkun, 1995).

Several components of the holoenzyme possess CTD kinase activity: TFIIH, DNA-PK and the SRB10/SRB11 or CDK8/cyclin C complex (Dvir et al., 1992; Lu et al., 1992; Peterson et al., 1992; Roy et al., 1994; Liao et al., 1995; Ossipow et al., 1995; Shiekhattar et al., 1995; Serizawa et al., 1995; Tassan et al., 1995). The presence of the SRB/SWI/SNF complexes in RNAP II holoenzymes confers a 12 fold greater phosphorylation of RNAP II (Kim et al., 1994). Mutants in SRB10/SRB11 show deficiencies in CTD phosphorylation *in vivo* (Liao et al., 1995). These CTD kinases are described in further detail in section A.2.

The yeast holoenzyme is exceptionally stable in the absence of DNA and is able to initiate efficient *in vitro* transcription when supplemented with TBP, TFIIE and DNA. It is thought that the preformed holoenzyme is recruited to promoters where TFIID is already bound (Koleske and Young, 1995). Regulation of transcription may occur during the association of TFIID to the promoter and during the binding of the holoenzyme to TFIID (Koleske and Young, 1995).

It is currently unknown how many different holoenzyme complexes are present in eukaryotes. A second yeast RNAP II holoenzyme containing RNAP II, TFIIF, and SRB proteins but not TFIIB or TFIIH has been isolated (Koleske and Young, 1995). Furthermore, a variety of different mammalian RNAP II holoenzymes exist. It will be interesting to see whether isolated holoenzymes are regulated differently or whether they exhibit cell type specificities (Koleske and Young, 1995; Goodrich et al., 1996).

PIC assembly by the multistep model may occur in parallel with the holoenzyme model. It is possible that the multistep process is more predominantly used for PIC formation because less than 20% of total cellular RNAP II is part of the holoenzyme in yeast, a fraction which may not be sufficient for transcription initiation on the 7000 genes in the yeast genome (Koleske and Young, 1995). Furthermore, the holoenzyme does not always contain a full set of GTFs, suggesting that even this method of assembly requires multiple steps of GTF incorporation into PIC (Maldonado et al., 1995). The holoenzyme model is favored when explaining synergistic activation by multiple activators. The large holoenzyme possesses a greater number of surfaces for simultaneous interaction with activators, enabling it to be a better binding partner than its individual components (Ossipow et al., 1995).

c. TATA-less promoters

A large number of class II genes appear to contain TATA-less promoters. These are mainly "housekeeping" genes which are active in all cells and are transcribed at a low rate, as compared to TATA-containing genes. TATA-less promoters require all GTFs, including TFIID and TFIIA, and require the pyrimidine-rich Inr element to accurately initiate transcription (reviewed in Goodrich et al., 1996; Roeder, 1996). TBP alone is not sufficient for directing transcription from TATA-less promoters; the TAFs are essential for TATA-less transcription (Goodrich et al., 1996).

Transcription mediated by TATA-less promoters involves specific interactions between proteins and the Inr element. These proteins may include TAFs and RNAP II (Roeder, 1996). Identified Inr-binding proteins include *Drosophila* $\text{TAF}_{\text{II}}150$, YY1, TFII-I, USF, and E2F. TBP-DNA contacts may be unimportant in Inr mediated transcription. Instead, Inr binding proteins may serve as potential nucleation sites for the entry of the rest of GTFs either by the multistep or holoenzyme models of PIC assembly (Roeder, 1996).

The requirement for TFIIA in Inr basal transcription may counteract TBP-interacting negative factors present in nuclear extracts and in TFIID preparations. Alternatively, TFIIA may stabilize weak interactions between TFIID and Inr elements or between TFIID and other GTFs (Roeder, 1996).

For promoters which contain both TATA and Inr elements, these sequences may cooperatively interact with the transcription apparatus in order to initiate transcription. Mutations in both TATA and Inr sequences result in greater decreases in transcription as compared to when either element is singularly mutated (Concino et al., 1984). It is possible that TATA and Inr sequences provide two anchorage points for PIC formation. TFIID binding to the TATA box and RNAP II binding to the Inr element (Carcamo et al., 1991) may impart greater PIC stability and an enhanced transcription rate (Zawel and Reinberg, 1993).

2. Initiation

The initiation of transcription follows PIC assembly at the promoter, and involves DNA duplex unwinding and synthesis of the first RNA phosphodiester bond. The initiated complex is termed an "open" complex. Transcription initiation requires ATP hydrolysis of the β-γ bond (Bunick et al., 1982; Sawadogo and Roeder, 1984), which occurs subsequent to PIC assembly and prior to the first phosphodiester bond formation. ATP hydrolysis is suspected to be involved in the establishment of "open" complexes (Jiang et al., 1993).

The TFIIH helicase activity is likely the source of ATP dependency. Two subunits of TFIIH, ERCC2 and ERCC3, are helicases which may be involved in "open" complex formation by melting the DNA between positions -9 to +1 (Holstege et al., 1996). These two subunits provide a bi-directional helicase activity where one subunit unwinds DNA in a 3' to 5' direction and the other subunit unwinds DNA in a 5' to 3' direction (Orphanides et al., 1996).

TFIIE may also play a role in "open" complex formation. At low concentrations *in vitro*, TFIIE stimulates the helicase activity of TFIIH (Serizawa et al., 1994). Other studies find that TFIIE represses ERCC3 helicase activity. ERCC2 helicase activity is unaffected by TFIIE (Zawel and

Reinberg, 1995). Some evidence suggests that TFIIE directly melts the DNA. TFIIF alone stimulates transcription to various degrees on supercoiled DNA templates (Orphanides et al., 1996). TFIIF may also function in "open" complex formation (Pan and Greenblatt, 1994; Robert et al., 1996).

3. Promoter clearance

Promoter clearance ensues in the presence of ribonucleoside triphosphates, after "open" complex formation. During promoter clearance, the activated complex begins to move down the DNA template and RNA synthesis begins. The melted region (transcription bubble) of the "open" complex moves downstream while upstream regions renature (Zawel and Reinberg, 1993; Orphanides et al., 1996; Zawel and Reinberg, 1995).

The shift from transcription initiation to elongation is accompanied by phosphorylation of the large subunit of RNAP II (Zawel and Reinberg, 1993; Zawel and Reinberg, 1995; Orphanides et al., 1996). RNAP II and the kinases that phosphorylate the protein are described in more detail in section A. It is unknown whether the phosphorylation of RNAP II has functional significance during the switch from transcription initiation to elongation. It has been hypothesized that the phosphorylation of RNAP II may directly contribute to the transition from transcription initiation to elongation. In support of this hypothesis, RNAP IIA but not RNAP IIO interacts with TBP and TFIIE, suggesting that the phosphorylation of the CTD may disrupt RNAP II-PIC interactions (Zawel and Reinberg, 1995). In addition, the SRB/SWI/SNF mediator associates with the RNAP II holoenzyme by binding to nonphosphorylated CTD. In this way, these chromatin remodeling and coactivating factors may be brought to the vicinity of the promoter. Phosphorylation of the CTD during the transition from initiation to elongation may release RNAP II from these initiation complexes. The newly phosphorylated CTD may then provide a platform for association of factors important for transcription elongation, such as elongation factors and RNA processing factors (Orphanides et al., 1996).

It is thought that some GTFs remain at the promoter poised for reinitiation while other GTFs proceed along with the elongating complex. *In vitro* studies with purified GTFs have shown that TBP or TFIID remains stably associated with the promoter after initiation and promoter clearance during transcription (Van Dyke et al., 1988; Van Dyke et al., 1989; Zawel et al., 1995; Orphanides et al., 1996). This facilitates reinitiation events even when other GTFs are dissociated from or remain associated with elongating RNAP II. TFIIB and TFIIE dissociate from RNAP II once RNAP II has advanced to nucleotide position +10. TFIIH also dissociates from RNAP II between nucleotide +30 to +68, whereas TFIIF may remain associated with elongating RNAP II either stably or transiently (reviewed in Roeder, 1996).

4. Elongation

Transcription elongation is thought to be catalyzed by fully phosphorylated RNAP IIO, and the conversion of IIA to IIO may facilitate the release of RNAP II from the PIC. This is supported by the observation that phosphorylation of RNAP II decreases its affinity for DNA promoters (Dahmus, 1994; Dahmus, 1996). The ternary elongation complex consists of the DNA template, nascent RNA, and RNAP II (and associated factors) (Zawel and Reinberg, 1995; Svejstrup et al., 1996).

Elongation by RNAP II is a discontinuous process, often consisting of long pauses and arrests at specific DNA sequences and at nucleoprotein complexes scattered throughout transcribed regions of eukaryotic genes (Reines et al., 1996). Purified RNAP II transcribes genes at 100 to 300 nucleotides per minute *in vitro*, whereas mRNAs are synthesized at 1500 to 2000 nucleotides per minute *in vivo*. The increased rate of transcription observed *in vivo* is facilitated by elongation factors which regulate the transcription elongation process by increasing the rate of RNAP II catalysis or by preventing elongation arrest (Aso et al., 1995a). To date, five general elongation factors have been identified. These include SII, TFIIF, the Elongin/SIII complex, positive transcription elongation factor (P-TEFb), and ELL (Reines et al., 1996).

Both SII and p-TEFb are involved in preventing RNAP II arrest by helping RNAP II pass through various nucleoprotein complexes and DNA sequences that act as intrinsic arrest sites. In brief, SII binds to and activates an endoribonuclease activity associated with RNAP II that cleaves nascent RNA transcripts present in paused complexes, resulting in a realignment of the RNA 3′ end with the catalytic site of RNAP II. This is described in further detail in the paragraph below. The mechanism by which P-TEFb prevents elongation arrest is unclear but involves the phosphorylation of the CTD of the large subunit of RNAP II (Marshall et al., 1996; Reines et al., 1996). P-TEFb phosphorylates purified RNAP II. In addition, P-TEFb is able to phosphorylate RNAP II when RNAP II is in an early elongation complex (Marshall et al., 1996). It is possible that all stages of elongation require fully phosphorylated RNAP II, which may become fully or partially dephosphorylated at pause sites. P-TEFb may facilitate elongation by phosphorylating incompletely phosphorylated or unphosphorylated forms of RNAP II back to IIO.

The inchworm model for elongation by RNAP II has been proposed to account for the observed 3′ to 5′ cleavage of RNA that follows transcription arrest. When RNAP II is arrested at an intrinsic arrest site or at some other impediment, RNAP II fails to translocate even though the nascent mRNA transcript continues to grow. The catalytic site then slips backward along the DNA and behind the 3′-hydroxy terminus of the RNA. In order to properly realign the catalytic site, cleavage upstream of the 3′-hydroxy terminus of the nascent RNA transcript creates a new 3′-hydroxy terminus that is properly positioned with respect to RNAP II and is capable of being extended. The active site for RNA cleavage resides in RNAP II. SII activates the RNAP II associated endonuclease responsible for this cleavage (reviewed in Aso et al., 1995a; Chamberlin, 1992-1993; Reines et al., 1996).

TFIIF, the Elongin/SIII complex, and ELL increase the overall rate of RNAP II transcription by suppressing transient pausing of the protein at many sites, other than intrinsic arrest sites, on the DNA template (Aso et al., 1995b; Reines et al., 1996). How these elongation general factors function is not well understood. Elongin/SIII complex may suppress pausing by maintaining proper positioning of the RNAP II catalytic site and the 3′-hydroxy end of the nascent RNA. The Elongin/SIII complex is composed of Elongin A, B, and C. Elongin A increases the catalytic rate of RNAP II at low levels by preventing transient pausing at various sites on DNA templates, whereas Elongin B and C are positive regulatory subunits which help upregulate Elongin A activity (Aso et al., 1995b; Reines et al., 1996). Both subunits of TFIIF, RAP30 and RAP74, are required for efficient elongation (Reines et al., 1996).

5. Termination

Termination involves the end of transcription and the release of the nascent RNA from the ternary elongation complex. This is distinct from elongation pausing, where RNAP II stops transcription but does not release its RNA transcript (Kerppola and Kane, 1991). Termination of RNAP II transcription occurs downstream of a polyadenylation (poly [A]) site. RNAP II continues to transcribe after the poly (A) sequence and is disengaged from the DNA at a number of different sites (Citron et al., 1984; Hagenbüchle et al., 1984; LeMeur et al., 1984; Rohrbaugh et al., 1985; Pribye et al., 1988; Maa et al., 1990).

The mechanisms for termination are not well understood. Termination appears to be facilitated by specific signals present in the DNA which may direct RNAP II to terminate transcription and release RNA. However, the role for intrinsic terminators of RNAP II have not been clearly defined (Richardson, 1993). With few exceptions, these DNA signals involve T-rich regions in the nontranscribed DNA strand. However, not all T-rich regions block RNAP II transcription, suggesting that other factors are involved in termination (reviewed in Kerppola and Kane, 1991).

Termination of RNAP II may also involve the recognition of other DNA sites near the site of termination (Dedrick et al., 1987). It has also been suggested that intrinsic termination is dependent on DNA structure. Termination may also occur when 3′ processing activities act on the nascent transcript to release the mRNA (Kerppola and Kane, 1990). Furthermore, termination sites may be dependent on accessory termination factors, including RNA, DNA and RNAP II binding proteins, that may aid this process (reviewed in Kerppola and Kane, 1991).

RNAP IIO must be dephosphorylated to regenerate RNAP IIA before reinitiation can occur. It is unknown when the conversion of IIA to IIO occurs. Dephosphorylation may occur immediately prior to, during or after the termination event. The phosphorylation state of RNAP II when it is released from the DNA template remains to be determined. It is possible that the dephosphorylation of the CTD, by a CTD phosphatase, is associated with the termination event (reviewed in Dahmus, 1994).

D. Regulation of RNAP II transcription

RNAP II transcription is tightly controlled by the combined regulatory effects of positive and negative factors, including activators, repressors and chromatin-associated protein. During the transcription of any given gene, a variety of signals dictate the end level of mRNA production (Goodrich et al., 1996).

1. Activation of transcription

Activated transcription involves the stimulation of transcription above basal levels and is facilitated by DNA-binding proteins, otherwise known as transcriptional activators. These proteins modulate the function of the GTFs and recognize gene-specific consensus sequences. Activators typically consist of a DNA-binding domain, that recognizes specific DNA elements present upstream or downstream of core promoter regions, and an activation domain, that is responsible for stimulating transcription. Activators are classified according to their amino acid composition. The herpes simplex virion protein VP16 is an acidic activator because its activation domain is rich in aspartate and glutamate residues. Other activation domains are proline or glutamine rich (Zawel and Reinberg, 1995).

The mechanisms by which activators stimulate transcription are currently under extensive investigation. Activators are capable of stimulating transcription from a variety of different promoters containing their recognized consensus DNA sequences. Different activators appear to affect different stages in transcription. Certain activators such as Sp1 stimulate initiation, while other activators such as the human immunodeficiency virus type 1 (HIV-1) Tat stimulate elongation. VP16 stimulates both initiation and elongation. Activators also function cooperatively or synergistically with other activators to enhance transcription (Blau et al., 1996). There may be several levels at which activators regulate gene transcription. These are described in the following sections.

a. By removing repressor proteins from promoters

It has been shown that nucleosomes present in the promoter prevent the efficient binding of GTFs and RNAP II (refer also to section D.2.d). As a result, chromatin must be remodeled in order to allow the GTFs and RNAP II to enter the promoter region. The process of chromatin remodeling is ATP-dependent and involves multisubunit complexes such as SWI/SNF. It is unclear as to how SWI/SNF complexes are recruited to promoters, although some activators may interact with proteins in the complex and the complex may be recruited by its association with the RNAP II holoenzyme. The SWI/SNF complex may possess some weak DNA-binding activity that might target these complexes to promoters. The recent isolation of yeast and mammalian holoenzymes that contain SWI/SNF components has altered this hypothesis (Orphanides et al., 1996).

Not all activators that remodel chromatin are associated with the holoenzyme. The mammalian activator LEF-1 binds DNA and is able to stimulate transcription when the enhancer is assembled into nucleosomal structures (Kingston et al., 1996).

Studies suggest that binding of an activator to a promoter can cause chromatin remodeling. This may be independent of interactions between activators and GTFs, since nucleosome disruption occurs in the absence of transcription initiation and elongation or when the TATA box is deleted. GAL4 derivatives which contain activation domains alleviate repression by nucleosomes when bound to promoters. It appears that the ability of an activator to bind stably in chromatin and activate transcription may not be linked. A stably bound factor lacking an activation domain may be able to bind to chromatin but still not carry out activated transcription. Although an activator may prevent chromatin repression by competing with histones at promoters, its activation domain may facilitate transcriptional activation by providing an entry site for other factors that are required for this process (Workman et al., 1991; Pugh, 1996).

In yeast, chromatin remodeling and transcriptional activation are dependent on the activator PHO4, which binds to two sites at the Pho5 promoter. Furthermore, the fusion protein containing the DNA binding domain of PHO4 and the activation domain of GAL11 (which is a component of the holoenzyme) efficiently recruits the RNAP II holoenzyme to the DNA in the absence of the classic activation domain of PHO4. As a result, chromatin is disrupted and transcription is activated. Similar chromatin remodeling is observed at the Pho5 promoter in the absence of the TATA box or the SWI/SNF complex, suggesting that the recruitment of the holoenzyme is sufficient for this event. Therefore, transcription initiation may not be required for chromatin disruption; instead, components of the transcription machinery may compete with nucleosomes for DNA binding (Gaudreau et al., 1997).

$TAF_{II}250$ possesses histone acetylation activity and may play an important role in promoting the access of activators and GTFs to DNA *in vivo*. Histone acetylation appears to be an important step in converting transcriptionally inactive chromatin to an active form. The acetylation of lysine residues in histones weakens their interaction with DNA. As a result of chromatin disruption, activators and GTFs are able to gain access to promoters (Tansey and Herr, 1997).

b. By recruiting transcription proteins to the promoter

Activators may function by interacting with other transcription proteins during the initiation stages of transcription. The activation domains of activators often are the regions for contact with the RNAP II basal transcription machinery (Goodrich et al., 1996). The GTFs that bind to eukaryotic activators include subunits of TFIID, TFIIB, TFIIF, and TFIIH (Zawel and Reinberg, 1995; Goodrich et al., 1996). For instance, VP16 interacts with TFIID (Stringer et al., 1990), TFIIB (Lin and Green, 1991), and TFIIH (Xiao et al., 1994). In addition, there is a correlation between the strength of VP16 to stimulate transcription and its ability to interact with TFIID (Ingles et al., 1991; Goodrich et al., 1996). Mutations in TFIIB that disrupt interactions between TFIIB and the transcriptional activation domain of VP16 disrupt activated transcription but not basal transcription (Roberts et al., 1993; Zawel and Reinberg, 1995; Goodrich et al., 1996).

Although activated transcription may require interactions between activators and GTFs, these interactions may not be sufficient for activation. Not all of these interactions may play a functional role in activation. VP16 may bind to TBP (Stringer et al., 1990) but recombinant TBP alone cannot participate in the activation process (Zawel and Reinberg, 1993; Zawel and Reinberg, 1995). The minimal set of transcription proteins required for basal transcription *in vitro* on most promoters include TBP, TFIIB, TFIIE, TFIIH, and RNAP II, but activated transcription requires coactivators, including TAFs (Verrijzer and Tjian, 1996; Tansey and Herr, 1997), TFIIA (Jacobson and Tjian, 1996), SRB/SWI/SNF mediator complexes (Björklund and Kim, 1996), and general coactivators (Kaiser and Meisterernst, 1996). Many of these coactivators complex to GTFs and activator proteins. The SRB/SWI/SNF mediator complex interacts with the acidic activation domains of VP16 (Peterson and Tamkun, 1995).

Although the TAF components of TFIID are dispensable for basal transcription, they are generally thought to be required for activated transcription. However, some studies suggest that TAFs are not required for transcriptional activation in yeast (Moqtaderi et al., 1996). To date, there are twelve known TAFs, ranging from 18 to 250 kDa, some of which interact with TFIIA, TFIIB, TFIIE, TFIIF and TFIIH (Roeder, 1996; Verrijzer and Tjian, 1996; Tansey and Herr, 1997). The TAFs target different classes of activators. For example, glutamine-rich Sp1 interacts with $TAF_{II}110$, acidic VP16 binds to $TAF_{II}40$ and $TAF_{II}60$, and isoleucine-rich NTF-1 interacts with $TAF_{II}150$ and $TAF_{II}60$. It has been suggested that distinct activators bind to specific TAFs in order to influence TFIID response on different promoters. These interactions may have functional significance since transactivation by Sp1 and NTF-1 is dependent on the presence of the TAFs that bind to these activators. In addition, mutations which disrupt interactions disrupt transactivation (Verrijzer and Tjian, 1996).

It is likely that the direct interaction between an activator and components of the RNAP II transcription apparatus occur early in the process of transcriptional activation. The mechanism by which these proteins carry out activated transcription has not been fully elucidated, but there are several existing models used to explain how protein-protein interactions enhance transcription levels from specific promoters. Through these interactions, activators may function to recruit GTFs to PICs.

There is evidence to show that activators recruit PIC components to the promoter. Activators recruit TFIID, TFIID/TFIIA, and TFIIB to DNA promoters. This recruitment correlates with the efficiency of the activator's transcriptional activation function and activator-GTF interactions. Regions important for interaction are also required for transcriptional activation (Goodrich et al., 1996). In addition, activator-TAF contacts may mediate TFIID, TFIID-TFIIA or TFIIB recruitment to the promoter (Verrijzer and Tjian, 1996). Contacts between activator and GTFs and/or TAFs may in turn facilitate and accelerate the assembly of the PIC formation (Zawel et al., 1995). These interactions may be especially important in directing transcription from promoters with weak core DNA elements (Goodrich et al., 1996).

It is possible that a single activator-holoenzyme interaction can recruit the entire RNAP II holoenzyme to the promoter (Barberis et al., 1995; Goodrich et al., 1996). Certain activators are components of the holoenzyme. The coactivator GAL11 protein, which may be involved in the transcriptional activation of various yeast genes, is present in the yeast holoenzyme. However, a mammalian holoenzyme isolated by Ossipow et al. (1995) is devoid of the transcriptional activators HNF-1, HNF-4 and C/EBPα.

Activators may also function to retain GTFs at the promoter after transcription initiation. Following promoter clearance, TFIID is the only GTF that is bound to the promoter. By recruiting and maintaining GTFs such as TFIIA and TFIIB, activators may facilitate multiple rounds of transcription by preventing GTF dissociation.

c. By inducing conformational changes in the PIC

Activator-GTF interactions may also function to stabilize the PIC by affecting the conformation of existing components assembled into PICs. In a multistep model, a conformational change may directly affect subsequent events in PIC assembly perhaps by improving the binding of another GTF (Goodrich et al., 1996). In the holoenzyme model, an activator induced conformational change may improve holoenzyme association at the promoter. Certain activators can alter the TFIID footprint on some promoters, suggesting that a conformational change has occurred. Activator-TAF interactions may stabilize the PIC on certain promoters, enhance TFIID binding to the core promoter and lead to a higher level of transcription (Verrijzer and Tjian, 1996). Conformational changes in TFIIB occur in the presence of the VP16 activation domain, which may stimulate subsequent TFIIF and RNAP II recruitment or enhance holoenzyme stability (Roberts and Green, 1994). In addition, the Epstein-Barr virus transactivator ZEBRA induces a conformational change in the TFIID-TFIIA complex, which may enhance transcription (Chi and Carey, 1996).

d. By inducing modifications to PIC proteins

Activators may also stimulate post-translational modifications to the GTFs on certain promoters. Although activators do not possess enzymatic activity, they may enhance the activity of protein kinases that associate with PICs. These kinases include the CDK7/MO15 subunits of TFIIH, CDK8/cyclin C pair associated with the mediator complex, DNA-PK, and $TAF_{II}250$. The first three kinases phosphorylate the large subunit of RNAP II. By promoting CTD phosphorylation and promoter clearance, activators may increase the transcription initiation rate. In support of this hypothesis, activators have been found to interact with TFIIH and the SRB mediator complex (Orphanides et al., 1996). $TAF_{II}250$ phosphorylates the RAP74 subunit of TFIIF (Dikstein et al., 1996). Phosphorylation of RAP74 may stimulate the initiation and elongation activities of TFIIF. There is evidence to suggest that phosphorylated RAP74 may facilitate efficient TFIIF assembly into DB-RNAP II/F complexes and TFIIF binding to RNAP

II (Kitajima et al., 1994). Phosphorylation of the RNAP II large subunit, RAP74 and possibly other GTFs may enhance transcription initiation and elongation (Goodrich et al., 1996).

e. By enhancing promoter clearance and elongation

Interactions between activators and GTFs may accelerate promoter clearance and/or elongation. As described by Goodrich et al. (1996), TFIIH may be involved in this type of activation. Activators such as VP16 which stimulate transcription elongation also interact with TFIIH (Blau et al., 1996). These contacts may facilitate promoter clearance by regulating TFIIH kinase and helicase activity. Efficient CTD phosphorylation and open DNA complex formation may be enhanced, resulting in an increased level of transcription from a target promoter (Goodrich et al., 1996).

Transcription elongation factors may increase transcription by facilitating RNAP II passage through various arrest sites (TFIIS and p-TEFb) or by stimulating the rate of RNA synthesis (TFIIF, Elongin/SIII, and ELL) (Aso et al., 1995a; Aso et al., 1995b). It is possible that activators may stimulate transcription elongation by recruiting these factors to the PIC and/or by enhancing their activities (Orphanides et al., 1996).

2. Repression of transcription

In recent years there has been considerable progress in identifying gene-specific transcriptional repressors and elucidating their modes of action. One major class of transcriptional repressors includes those that bind to DNA and interact with gene-specific promoter sequences. In contrast to activators, each of these repressors contains a repression domain, which can be categorized according to its primary amino acid sequence. For instance, repressors containing alanine-, glutamine-, proline-, charged-, and hydrophobic-rich domains have been identified. Some of these repression domains appear to be critical for function (Johnson, 1995; Hanna-Rose and Hansen, 1996).

Interestingly, certain repressor proteins are found directly in the RNAP II holoenzyme. For instance, SIN4 and RGR1, repress transcription of many yeast genes and are components of the yeast RNAP II holoenzyme (Orphanides et al., 1996).

Transcriptional repression may be mediated by protein-protein interactions between repressor molecules and the basal and activated transcription machinery. Repressor targets include GTFs, activators or coactivators, and corepressors. Corepressors serve as bridger molecules between repressors and their targets. Examples of corepressors are the eukaryotic SIN3A and SIN3B proteins, which are corepressors for MAD and MXI1 mediated repression (MAD-MAX or MXI1-MAX heterodimers) (Hanna-Rose and Hansen, 1996).

a. Direct repression

Direct repression occurs when the formation or activity of the PIC is inhibited by direct interactions between repressor proteins and the GTFs or with RNAP II. Repressors which interact with the GTFs are thought to repress a minimal RNAP II promoter which contains only a TATA or Inr element. An example of this type of repressor is the eukaryotic repressor TR. Repression of the basal RNAP II transcription machinery through interactions with GTFs may sterically block the addition of other proteins, thereby affecting PIC assembly and transcription initiation. Other interactions between repressors and GTFs may have direct effects on transcription elongation (Hanna-Rose and Hansen, 1996).

In some cases, repressors do not bind to DNA directly but bind to DNA bound proteins. An example of this type of repressor is Dr-1, which does not bind to DNA but only interacts with components of the basal transcription machinery. The alanine- and glutamine-rich repressor domains of Dr-1 are essential for inhibitory function (Zawel and Reinberg, 1995; Hanna-Rose and Hansen, 1996). The mechanisms by which Dr-1 functions are twofold. Complexes of TFIID and Dr-1 bound to the TATA box are not recognized by TFIIB. Moreover, Dr-1 is able to disrupt preformed DAB-RNAP II/F complexes. As a result, basal transcription is repressed (Zawel and Reinberg, 1995). Activators may relieve repression by binding to repressor proteins. The adenovirus E1A protein and the SV40 large T antigen prevent or displace TBP-Dr-1 interactions (Drapkin et al., 1993; Kraus et al., 1994). In addition, TFIIA can relieve the repressive effects of Dr-1 (Inostroza et al., 1992).

Direct repression also includes interactions between repressors and corepressors which target the basal RNAP II machinery and may involve the assembly of PICs that are inactive (Hanna-Rose and Hansen, 1996).

b. Quenching

Quenching involves interference with the transcriptional stimulatory activity of an activator that is bound to the promoter. During quenching, only specific promoters and activators are repressed; basal transcription is unaffected. Quenching is accomplished by protein-protein interactions between a repressor and a specific activator or coactivator. As a result of the above interactions, important contacts between an activator or coactivator with its target may be prevented. As a result, activated transcription is repressed while basal transcription continues (Hanna-Rose and Hansen, 1996).

c. Protein-DNA interactions

Repression may also be the direct result of protein-DNA interactions that lead to steric hindrance. The *Drosophila* P-element transposase binds to the TATA region of the P-element promoter. As a result, RNAP II and GTFs are unable to gain access to this region (Zawel and Reinberg, 1993; Zawel and Reinberg, 1995). Binding of repressors to DNA may exclude the binding of an activator (Johnson, 1995).

d. Chromatin mediated repression

The repression of transcription may in part be chromatin mediated. Nucleosomes interfere with the formation of PICs at promoters, with the binding of activators, and with the processes of transcription, initiation and elongation. It is thought that the formation of PICs and the association of activators is in direct competition with nucleosome assembly on DNA (Kingston et al., 1996). Nucleosomes may repress PIC assembly and block transcription initiation by preventing the binding of transcription factors to DNA, either through steric hindrances or distortion of target binding sites (Edmondson and Roth, 1996; Paranjape, 1994). In addition, transcription elongation is inhibited on nucleosomal templates (Izban and Luse, 1991; Izban and Luse, 1992; Kingston et al., 1996).

It is possible that repressors recruit complexes that stabilize nucleosomes. In yeast, the SSN6 and TUP1 proteins repress the α-specific genes that are derepressed under specific growth conditions. Neither SSN6 nor TUP1 bind to DNA but are targeted to specific promoters by other factors such as α2/MCM1. These proteins fractionate from yeast cells in a large complex, suggesting that they are involved in the formation of repressive complexes on chromatin. In addition, chromatin structures are present under SSN6 and TUP1 repressive conditions (Kingston et al., 1996).

Activators may play a role in relieving chromatin mediated repression by a variety of mechanisms (refer also to section D.1.a). Activators may stimulate transcription by binding to DNA and preventing nucleosome formation. This has been defined as antirepression (Croston et al., 1991). For example, sequence specific activators, such as Sp1 and GAL4-VP16, counteract histone mediated repression (Croston et al., 1991). Activators may also form contacts with PIC components, thereby recruiting these proteins to the promoter and inhibiting nucleosome assembly (Kingston et al., 1996). Alternatively, activators may compromise nucleosome stability by binding to a site within the nucleosome (Edmondson and Roth, 1996).

Nucleosomal repression may also be relieved by proteins which remodel chromatin and specifically disrupt nucleosomal structures. The SWI/SNF genes of yeast encode proteins which modify chromatin in an ATP-dependent manner during transcriptional activation. The holoenzyme, the SWI/SNF complex, and the SRB/SWI/SNF complex are able to disrupt nucleosomes. This may facilitate PIC assembly onto promoters. The holoenzyme enhances TBP/TFIIA binding onto a nucleosomal template in the presence of ATP (Kingston et al., 1996).

E. Herpes simplex virus type 1 (HSV-1)

Herpes simplex virus type 1 (HSV-1) belongs to the Herpesviridae family and the α-herpesvirus subfamily. A short list of members from the α-, β-, and γ-herpesvirus subfamilies is shown in Table 1.1. Herpesviruses are categorized on the basis of their biological properties and DNA sequences. In general, the α-herpesviruses possess a variable host range and a short reproductive growth cycle. Latent α-herpesvirus infections are primarily localized in sensory ganglia. The β-herpesviruses display a restrictive host range and a long reproductive growth cycle. Latent β-herpesvirus infections are maintained in secretory glands, lymphoreticular cells, kidneys and other tissues. The γ-herpesviruses also show a restrictive host range; members of this subfamily are generally specific for either T or B lymphocytes. Duration of the γ-herpesvirus reproductive cycle is variable and latent infection occurs most frequently in lymphoid tissues (Roizman et al., 1981; Roizman and Sears, 1990). HSV-1 is a human neurotropic DNA virus that is often used as a prototype for understanding the pathogenesis and molecular biology of herpesviruses.

1. Viral DNA

The HSV-1 DNA is packaged in the form of a toroid (Furlong et al., 1972) and is not assembled into nucleosomes (Muggeridge and Fraser, 1986; Deshmane and Fraser, 1989). The HSV-1 genome is a linear double-stranded DNA molecule (approximately 152 kb) that has a G + C content of approximately 68% (Kieff et al., 1971). The viral genome contains two components of DNA, long (U_L) and short (U_S) regions, which are covalently linked (Fig. 1.1). Each region contains unique sequences flanked by inverted terminal repeats. The repeats of the U_L region are designated as ab and b'a', whereas the repeats of the U_S region are designated as a'c' and ca (Fig. 1.1). During viral DNA replication, the U_L and U_S regions of HSV-1 invert relative to one another, resulting in one of four isomers. This process is called isomerization. The DNA in any single virion particle exists in one of four configurations (Fig. 1.1A to 1.1D). Equimolar amounts of viral progeny containing each isoform are produced after infection (reviewed in Roizman and Sears, 1990). HSV-1 encodes more than 75 genes (Roizman and Sears, 1990), some of which are shown in Fig. 1.2 (McGeoch et al., 1988).

2. Virion

The HSV-1 virion consists of a core containing viral DNA, an icosadeltahedral capsid surrounding the core, an amorphous tegument surrounding the capsid and an outer envelope with projections (spikes) on its surface. There are at least 33 viral proteins present in the virion particle, many of which are glycoproteins. Some of these glycoproteins are components of the envelope spikes (reviewed in Roizman and Sears, 1990).

3. Viral replication overview

An HSV-1 primary infection is usually in oral mucosal epithelial cells and occurs through virion-cell contact. Productive infection may lead to tissue damage and an induction of the host inflammatory response, resulting in painful vesicular lesions. HSV-1 initiates infection by attaching to host cell receptors after which the viral envelope fuses to the cell membrane. The capsid is then transported to the nuclear pores and the DNA is released into the nucleus. After the DNA enters the nucleus, it immediately circularizes (Roizman and Sears, 1990).

After a primary infection, HSV-1 enters either a lytic or latent cycle. During the latent cycle, the virus is in a silent state where most viral gene transcription is suppressed. Latent infection will be described in further detail later in this chapter (refer to section E.8). During the lytic cycle, viral transcription, viral DNA replication and assembly of new capsids occurs in the nucleus of the host cell. Transcription of viral genes occurs throughout the lytic cycle and is dependent on the host's RNAP II transcription machinery, such that HSV-1 diverts the cellular transcription apparatus away from expression of the host genome and toward expression of the viral genome. The HSV-1 genes are tightly controlled and are expressed in a sequentially ordered temporal cascade, which is controlled by viral regulatory proteins that either enter with the virion particle or are synthesized early in infection (Roizman and Sears, 1990; Smiley et al., 1991).

Viral DNA replication ensues after the synthesis of specific viral encoded DNA replication enzymes and DNA binding proteins. Viral DNA synthesis occurs by a rolling circle mechanism, producing head-to-tail concatemers that are cleaved and packaged into capsids (Roizman and Sears, 1990).

The formation of viral progeny occurs in several steps. DNA is packaged into preassembled capsids. The virus matures and buds through the lamellae of the nuclear membrane, acquiring additional proteins and an envelope in the process. The enveloped capsids egress out of the cell and into the extracellular space through the endoplasmic reticulum (Roizman and Sears, 1990).

4. HSV-1 temporal gene expression cascade

In a productive lytic infection, the HSV-1 genes are expressed in a tightly controlled temporal cascade, which is regulated primarily at the transcriptional level (Fig. 1.3). The HSV-1 genes are classified into three groups according to their order of expression. The synthesis of proteins within each group has similar requirements and kinetics of expression (Roizman and Sears, 1990).

The immediate-early (IE or α) genes are the first to be expressed and are transcribed immediately after infection. IE genes encode five proteins: the infected cell polypeptide (ICP)0, ICP4, ICP22, ICP27, and ICP47. IE gene expression does not require viral protein synthesis but is instead stimulated by the virion tegument component VP16 in complex with host cell transcription factors, including octamer transcription factor (OCT-1). VP16 is released into the cell after fusion of the viral envelope with the cell membrane and is then transported to the nucleus (reviewed in Roizman and Sears, 1990). Maximum IE protein synthesis occurs between 2 and 4 hr post-infection, but IE proteins continue to accumulate late in infection (Honess and Roizman, 1974).

Four of five IE gene products (ICP0, ICP4, ICP22, and ICP27) are regulatory proteins which are involved in the subsequent expression of the delayed-early (DE or β) and late (L or γ) genes (Roizman and Sears, 1990). The fifth IE gene product ICP47 inhibits antigen presentation to $CD8^+$ T lymphocytes in infected human fibroblasts (York et al., 1994).

Transcription of DE genes is dependent on the presence of ICP4 (DeLuca and Schaffer, 1985; Godowski and Knipe, 1986). The DE genes encode proteins which are synthesized maximally between 5 and 7 hr post-infection (Honess and Roizman, 1974) and are involved in nucleic acid metabolism and viral DNA synthesis. These proteins include the major DNA binding protein, ICP8, the viral thymidine kinase and the viral DNA polymerase. DE protein production marks the beginning of viral DNA synthesis (Roizman and Sears, 1990), which is detected as early as 3 hr post infection and continues to 12 and 15 hr post-infection (Roizman et al., 1965).

The L genes are expressed after DE genes and are dependent on viral DNA replication to various degrees. L proteins are synthesized maximally after 5 hr post-infection. There are two subclasses of L gene, leaky L (γ1) and true L late (γ2), depending on whether they are partially (leaky L) or wholly (true L) dependent on viral DNA replication for their expression. L genes are dependent on the presence of ICP4 (Watson and Clements, 1980; DeLuca et al., 1984) and ICP27 (Sacks et al., 1985) for their expression and encode structural proteins, including glycoprotein B, glycoprotein D, and the major capsid protein ICP5.

5. Regulation of viral gene expression

a. Structure of HSV-1 genes and mRNA

Host cell RNAP II is responsible for transcribing HSV-1 genes (Costanzo et al., 1977), which are similar to cellular genes in structure, possessing typical RNAP II promoter and polyadenylation sequences. One difference between cellular and viral genes is that most viral genes do not contain intronic sequences (Roizman and Sears, 1990). Minimal promoter domains for all HSV-1 genes include a TATA box and transcription initiation start site; however, some TATA-less HSV-1 genes exist (Chou and Roizman, 1986).

All IE genes are transcriptionally activated by VP16 and associated factors acting at the *cis*-acting promoter element, 5' NC GyATGnTAATGArATTCyTTGnGGG 3' (N = any base, r = purine, and y = pyrimidine), located 150 to 300 bp upstream of the transcription initiation site of each IE gene (Mackem and Roizman, 1982; Kristie and Roizman, 1984). Multiple Sp1 sites are present in the G + C rich 5' nontranscribed region of the ICP4 gene, although Sp1 binding sites are not present in other IE gene promoters (Roizman and Sears, 1990). Other regulatory sequences include a high affinity ICP4 canonical binding site, ATCGTCNNNNyCGrC (Faber and Wilcox, 1986), present in the ICP4 and ICP0 genes (Kristie and Roizman, 1986; Kristie and Roizman, 1987; Faber and Wilcox, 1988).

The sequence requirements and activators that regulate DE and L gene expression are not well understood. Some binding sites for cellular transcription factors, such as Sp1 (Coen et al., 1986; Johnson and Everett, 1986; McKnight and Tjian, 1986; Homa et al., 1988), are present in DE and L genes. However, discrete *cis*-acting sequences conserved in DE or L genes, which bind viral regulatory proteins and activate transcription, have not been clearly identified. DE promoters consist of proximal upstream elements linked to TATA and cap site sequences, whereas L promoters consist of a TATA box and sequences surrounding the cap site.

Of particular interest are the putative ICP4 binding sites present in some DE and L genes, which often deviate from the canonical ICP4 binding sequence found in ICP4 and ICP0 promoters. Putative ICP4 binding sites are present in the thymidine kinase gene, upstream and downstream of the cap site. The functions of these sites are not clear. Many DE and L genes lack ICP4 binding sites in their promoters, and in one DE promoter which does have ICP4 binding sites, these sites can be removed without consequence (Smiley et al., 1992).

Viral mRNAs are capped and polyadenylated, but only a small number of mRNAs are processed by splicing (Roizman and Sears, 1990). Those mRNAs that are spliced are mainly IE gene products, such as ICP0 (Spatz et al., 1996) and ICP22 (Whitton and Clements, 1984).

b. VP16

VP16 (also called *trans*-inducing factor (α-TIF), Vmw65, and ICP25) activates transcription of IE gene promoters after associating with an accessory protein termed host cell factor (HCF) (Wilson et al., 1993). The binding of HCF to VP16 activates VP16 for association with another host protein (Wilson et al., 1993), the octamer transcription factor (OCT-1) (Kristie and Roizman, 1987; Kristie and Roizman, 1988; O'Neill et al., 1988). The VP16-HCF complex does not bind directly to viral DNA but instead binds to the complex formed between OCT-1 and the cis-acting 5' NCGyATGnTAATGArATTCyTTGnGGG 3' sequence (Mackem and Roizman, 1982; Kristie and Roizman, 1984) in IE promoters (McKnight et al., 1987; Gerster and Roeder, 1988; Preston et al., 1988; Triezenberg et al., 1988a; O'Hare and Goding, 1988). The acidic C terminal domain of VP16 is responsible for its transactivation function (Sadowski et al., 1988; Triezenberg et al., 1988b).

c. Immediate-early (IE) regulatory proteins

i. ICP0

ICP0 (also called Vmw110) is a nuclear phosphoprotein of approximately 110 to 120 kDa (Everett et al., 1991b). ICP0 contains an acidic N terminal region, two central proline rich regions and an N terminal cysteine rich zinc finger domain, known as the RING finger. RING finger motif proteins are thought to play a role in transcription, recombination, and signal transduction, by interacting with both DNA and other proteins. ICP0 binds nonspecifically to DNA *in vitro* (Hay and Hay, 1980; Everett et al., 1991b) and to chromatin *in vivo* (Hay and Hay, 1980).

In transient transfection assays, ICP0 acts as a potent and promiscuous transactivator of gene expression from class II promoters (Cai and Schaffer, 1991; Cai and Schaffer, 1992). Surprisingly, transactivation by ICP0 is independent of any *cis*-acting elements, including the TATA box, in target gene promoters (Nabel et al., 1988; Chen and Silverstein, 1992). During transient transfection assays, ICP4 elicits a synergistic activation of reporter genes along with ICP0 (Everett et al., 1991b). It is surprising that only one gene, ICP6, which encodes the large subunit of ribonucleotide reductase, requires ICP0 for expression in a lytic infection in tissue culture cells (Desai et al., 1993).

The function of ICP0 may be mediated by protein-protein interactions with other cellular and viral proteins. ICP0 forms multimers in solution (Everett et al., 1991a; Chen et al., 1992) and interacts with ICP4 (Yao and Schaffer, 1994; Mullen et al., 1995) and a 135 kDa cellular protein named herpesvirus-associated ubiquitin-specific protease (HAUSP), which is a novel member of the ubiquitin-specific protease family (Meredith et al., 1994; Meredith et al., 1995; Everett et al., 1997). It is possible that interactions between ICP4 and ICP0 serve to stabilize the binding of ICP4 and/or ICP0 to DNA, resulting in both activators stimulating gene transcription synergistically (Yao and Schaffer, 1994).

Although most ICP0 is nuclear, the protein can be detected in the cytoplasm of infected cells. ICP0 has been found to interact with the translation elongation factor 1δ (EF-1δ) (Kawaguchi et al., 1997). The domain of ICP0 required for EF-1δ interaction appears to inhibit translational efficiency in a rabbit reticulocyte lysate *in vitro* (Kawaguchi et al., 1997). These studies suggest that ICP0 performs multiple activities during lytic infection and may not only function in the transactivation of genes but may play a direct role in protein synthesis.

The multiplicity of infection (MOI) used to infect cells appears to dictate the role of ICP0 during lytic infection. At low MOIs (< 1 PFU/cell), ICP0 mutant viruses grow poorly and synthesize low levels of DE and L mRNAs and proteins. In contrast, at high MOIs (5 to 10 PFU/cell), ICP0 mutant viruses grow well and synthesize WT levels of gene products (Sacks and Schaffer, 1987; Everett et al., 1991b). These results indicate that at high MOIs, ICP0 is not required for efficient expression of viral genes. It has been suggested that during high MOI infections, high levels of VP16 stimulate IE gene expression to a degree that overcomes the need for ICP0. It is possible that VP16 and ICP0 perform complementary roles in viral gene transcription (Everett et al., 1991b; Cai and Schaffer, 1992).

ICP0 appears to play a critical regulatory role in the absence of virion components since cells transfected with ICP0 mutant virus DNA show reduced levels of virus production. In addition, virus production is delayed for 2 days. Cotransfection of an ICP0 expressing plasmid with the ICP0 mutant virus DNA complements the defect (Cai and Schaffer, 1989).

Other experiments provide evidence for ICP0's transactivation functions. Viral DNA that has been induced into a dormant, latent-like state can be reactivated into a lytic cycle by superinfection with wild-type (WT) HSV-1, with viruses mutant in any IE gene except ICP0, and with an adenovirus recombinant expressing ICP0 (Russell and Preston, 1986; Russell et al., 1987; Harris et al., 1989; Everett et al., 1991b). ICP0 appears to play a essential role, in the absence of other viral proteins, in the reactivation from viral latency. This is also observed *in vivo*, where ICP0 is able to promote reactivation from neuronal latency in some cases (Leib et al., 1989). There is a correlation between the transactivation efficiency and the reactivation capability of ICP0 mutants (Cai et al., 1993).

It has been suggested by Everett et al. (1991b) that ICP0 acts directly on the latent virus genome. As a result, chromatin proteins may be displaced, allowing the RNAP II transcription apparatus to assemble on latent viral DNA. Latent DNA, unlike lytic DNA, is assembled into nucleosomes as in cellular chromatin (Muggeridge and Fraser, 1986; Deshmane and Fraser, 1989).

ii. ICP4

ICP4 (also called Vmw175), of approximately 160 to 170 kDa, is localized to the nucleus (Courtney and Benyesh-Melnick, 1974) and exists in solution as a homodimer (Metzler and Wilcox, 1985). The ICP4 protein contains transactivation and DNA binding domains (DeLuca and Schaffer, 1988; Shepard et al., 1989), and is post-translationally modified by phosphorylation on serine and threonine residues (Pereira et al., 1977; Wilcox et al., 1980; Metzler and Wilcox, 1985). Other post-translational modifications on ICP4 include poly-ADP-ribosylation (Preston and Notarianni, 1983; Blaho et al., 1992).

ICP4 is the major viral transcriptional activator and is essential for virus growth and for DE and L gene transcription (Watson and Clements, 1980; DeLuca et al., 1985). Mutational analyses have identified acidic domains in the ICP4 protein that are necessary for its transactivation activity (Everett, 1984; DeLuca et al., 1985; Gelman and Silverstein, 1985), but the mechanism by which ICP4 transactivates DE and L gene promoters is not well understood. ICP4 binds nonspecifically to DNA (Freeman and Powell, 1982). The protein has some specificity in DNA binding activity for the sequence ATCGTCNNNNyCGrC (r = purine, y = pyrimidine, N = any base) (Faber and Wilcox, 1986). The transactivation functions of ICP4 may be regulated through ICP4's DNA binding to 5' upstream regulatory regions in some genes. There is a strong correlation between ICP4 mutants that cannot bind DNA and the loss of the protein's transactivation function (Paterson and Everett, 1988).

However, the ability of ICP4 to bind DNA has not been clearly shown to contribute to transactivation (Beard et al., 1986; Imbalzano, 1990). In some cases where ICP4 induces the expression of a gene, deletion of DNA sequences for which ICP4 shows high affinity does not appear to affect activation by ICP4 (Smiley et al., 1992). Mutant ICP4 proteins with reduced affinity for DNA retain the ability to stimulate certain DE and L genes (Shepard et al., 1989). Many studies have failed to identify ICP4 specific induction sequences in most, if not all, of the viral gene promoters transactivated by ICP4 (Eisenberg et al., 1985; Coen et al., 1986). ICP4 is able to transactivate the transcription of minimal promoters in which the only recognizable *cis*-acting element is the TATA box (Imbalzano et al., 1991). It is possible that ICP4 transactivates by binding to the target template at substantial distances from the site of ICP4 action in a manner that is characterized by low affinity and high degeneracy with respect to sequence specificity (Smith et al., 1993).

Not only does ICP4 transactivate genes, but it also has the ability to repress the transcription of the ICP4 and ICP0 genes (Dixon and Schaffer, 1980; DeLuca and Schaffer, 1985; Resnick et al., 1989). In the case of the ICP4 gene, the ICP4 consensus binding site overlaps the transcription start site (DeLuca and Schaffer, 1985). Therefore, transcription of the ICP4 gene may be down-regulated by ICP4's binding to this site. Whether ICP4 represses or transactivates a gene may be determined by the position of ICP4 binding on the DNA or the strength of this binding (Roizman and Sears, 1990).

Differential phosphorylation of ICP4 may also play a specific role in the regulatory functions of the protein. There are at least three forms of ICP4 detected on denaturing polyacrylamide gels (Pereira et al., 1977), all of which are post-translationally modified by phosphorylation (Pereira et al., 1977; Wilcox et al., 1980; Ackermann et al., 1984). The predominant form of ICP4 present depends on the particular stage in the lytic life cycle (Wilcox et al., 1980). In addition, the presence of ICP27 affects the modification of ICP4 (Rice and Knipe, 1988). In one study performed by Papavassiliou et al. (1991), phosphorylation of ICP4 was not required for the protein's binding to high affinity sites present in IE promoters, but instead enhanced ICP4's binding to noncanonical sequences present in DE and L promoters. Another study performed by Michael et al. (1988) showed that fully phosphorylated ICP4 binds to high affinity sites but not noncanonical sites. Despite the discrepancies in these results, the ability of ICP4 to bind to DNA sites in IE, DE and L genes may be dependent on the extent of phosphorylation (Michael et al., 1988; Papavassiliou et al., 1991).

Some studies suggest that ICP4 function is mediated by its interactions with other cellular and viral proteins and is not solely dependent on its binding to DNA. *In vitro*, ICP4 forms a tripartite complex with TBP and TFIIB on DNA (Smith et al., 1993), suggesting that ICP4 may aid in PIC formation on viral promoters. In the presence of all three proteins, TBP and ICP4 possess higher affinity for their respective binding sites, indicating that PIC assembly may be enhanced by improved protein-DNA binding (Smith et al., 1993). ICP4 also associates with the coactivator $TAF_{II}250$, an interaction which may be essential for activated transcription (Carrozza and DeLuca, 1996).

ICP4 also interacts with ICP0 (Yao and Schaffer, 1994; Mullen et al., 1995; Panagiotidis et al., 1997) and ICP27 (Panagiotidis et al., 1997). These interactions may be necessary for ICP4's transactivation activities. It is possible that ICP4 protein-protein interactions help modulate the ability of ICP4 to form complexes on DNA at lower affinity sites. ICP27 preferentially binds to less modified forms of ICP4 and is able to form a complex with ICP4 bound to a noncanonical binding site present in the HSV-1 thymidine kinase gene. ICP27 may modulate the DNA binding activity of ICP4 by affecting ICP4's post-translationally modified state (Panagiotidis et al., 1997).

In support of this hypothesis, ICP4's electrophoretic mobility is altered in the absence of ICP27 (Rice and Knipe, 1988). The interactions between IE proteins may aid in coordinately regulating transcription on certain viral genes.

The ICP4 protein also interacts with Epstein-Barr virus small RNAs (EBER)-associated protein (EAP) (Leopardi and Roizman, 1996), a host nucleolar protein, which associates with ribosomes (Toczyski et al., 1994) and binds to small RNAs of unknown function in Epstein-Barr virus infected cells (Toczyski and Steitz, 1991; Toczyski and Steitz, 1993). The function of EBERs and EBER-EAP complexes is unknown. Furthermore, the significance of EAP and ICP4 interaction has not been determined.

iii. ICP22

ICP22 (also called Vmw68) is a 70 to 82 kDa, 420 amino acid nuclear phosphoprotein (Purves and Roizman, 1992). Little is known about the structural features of ICP22. However, amino acid sequence analysis of the ICP22 protein reveals distinct domains. Amino acids 161 to 292 of HSV-1 ICP22 are highly conserved amongst ICP22 homologues from other herpesviruses, including bovine herpesvirus, equine herpesvirus, pseudorabies herpesvirus, varicella-zoster virus and Marek's disease virus (Schwyzer et al., 1994). In HSV-1 ICP22, amino acids 292 to 386 contain predominantly acidic and hydroxyl residues, whereas amino acids 387 to 420 contain mainly basic and proline residues (Schwyzer et al., 1994). There are 45 serine and 28 threonine residues in the ICP22 protein, the majority of which occur near the N terminus between amino acids 38 and 118 and the C terminus between amino acids 296 and 385. These particular regions may be potential sites for ICP22 phosphorylation (Purves and Roizman, 1992).

ICP22 is post-translationally modified by phosphorylation. WT patterns of ICP22 phosphorylation are dependent on the presence of the viral kinases US3 and UL13 (Purves and Roizman, 1992; Purves et al., 1993). ICP22 has also been shown to be nucleotidylylated *in vitro* (Blaho et al., 1994; Mitchell et al., 1994). Recent reports indicate that ICP22 is localized to the nucleus; the protein displays some preferential localization into DNA replication compartments (Leopardi et al., 1997).

ICP22 activity during lytic infection has not been well characterized. ICP22 is not essential for viral DNA replication in cell culture, but ICP22 mutants show impaired growth in certain cell lines (rabbit skin, baby hamster kidney [BHK], RAT-1, and human embryonic lung [HEL] cells) (Sears et al., 1985; Purves et al., 1993). In a nonpermissive infection, ICP22 mutants display a prolonged expression of some DE proteins and reductions in L proteins, suggesting that ICP22 has some regulatory functions (Astor, Unpublished data; Sears et al., 1985; Poffenberger et al., 1993). Levels of ICP0 and several late proteins are reduced in a restrictive ICP22 mutant infection (Purves et al., 1993). ICP22 functions may be complemented by some host factor in permissive cells, such as Vero cells.

Transcription of the ICP22 gene yields two mRNAs of different sizes. The longer transcript encodes the full-length 420 amino acid protein, while the shorter transcript encodes a smaller protein of 273 amino acids that is identical to the C terminus of the full-length ICP22 protein (amino acids 147 to 420). The shorter mRNA initiates transcription at +954 in the ICP22 gene; the function of the shorter ICP22 protein is currently undefined (Carter and Roizman, 1996).

The ICP22 protein interacts with EAP (Leopardi, 1997). EAP associates with ribosomes (Toczyski et al., 1994), binds to small RNAs of unknown function that are produced in Epstein-Barr virus infected cells (Toczyski and Steitz, 1991; Toczyski and Steitz, 1993), and interacts with ICP4 (Leopardi and Roizman, 1996). ICP22 interactions with EAP are independent of ICP4. EAP may serve as a molecular bridge facilitating ICP4 and ICP22 interactions with each other or with additional proteins (Leopardi et al., 1997).

iv. ICP27

ICP27 (also called Vmw63) is a 63 kDa nuclear phosphoprotein (Ackermann et al., 1984; Knipe et al., 1987). The protein possesses a 15 amino acid stretch that consists entirely of glycine and arginine residues. This region resembles a RGG box, a motif found in RNA binding proteins which play a role in mRNA and rRNA metabolism (Mears et al., 1995). Recent studies demonstrate that ICP27 is methylated *in vivo* (Mears and Rice, 1996).

The formation of infectious viral progeny in cell culture is dependent on the presence of ICP27 (Sacks et al., 1985; Rice and Knipe, 1988; McCarthy et al., 1989; Rice et al., 1993). ICP27 null mutants display distinct phenotypes. These mutants are deficient in L gene and protein expression (Sacks et al., 1985; McCarthy et al., 1989; Rice and Knipe, 1990) and show a five to twenty fold reduction in viral DNA synthesis (Rice and Knipe, 1990).

ICP27 is a multifunctional regulatory protein that appears to play roles in transcription, DNA replication, and mRNA processing. ICP27 has both positive and negative effects on different target DE and L promoters during transient transfection assays (Everett, 1986; Su and Knipe, 1987; Rice and Knipe, 1988; Sekulovich et al., 1988). Expression of ICP27 can stimulate chloramphenicol acetyltransferase (CAT) expression under the control of the glycoprotein B promoter (Rice and Knipe, 1988). However, in most transient transfection assays, the presence of ICP27 has little effect on HSV-1 promoters (Everett, 1984; DeLuca et al., 1985; Sekulovich et al., 1988). ICP27 either inhibits or augments the combined stimulatory activities of ICP0 and ICP4 on DE and L gene promoters, when ICP27 is cotransfected with both ICP0 and ICP4 into cells (Su and Knipe, 1987; Sekulovich et al., 1988; Rice et al., 1989). In these experiments, down-regulation of the ICP27 (Gelman and Silverstein, 1987) and DE ICP8 (Rice et al., 1989) promoter driven reporter genes occurs when ICP27 is expressed with ICP0 and ICP4. Furthermore, ICP27 enhances ICP0 and ICP4 induced expression from L ICP5 (Everett, 1986) and glycoprotein B (Rice and Knipe, 1988) promoter driven reporter genes. In summary, results from these transient transfection assays suggest that ICP27 may be involved in modulating the activities of ICP0 and ICP4 (Rice and Knipe, 1988; Sekulovich et al., 1988; McMahan and Schaffer, 1990). It is not clear from these cotransfection assays whether ICP27 is acting at transcriptional or post-transcriptional levels.

ICP27 may regulate virus gene expression at the transcription level. Nuclear run-on assays were performed to measure the transcription rates of various viral genes in ICP27 mutant infected cells. Transcription of the leaky (γ1) L genes is reduced and the true (γ2) L genes are not transcribed in an ICP27 mutant infection (McCarthy et al., 1989). The results of these experiments differ from those reported by Smith et al. (1992), who show that L gene transcription is not reduced in ICP27 mutant infected cells. In spite of WT levels of gene transcription, L mRNA products are of low abundance in ICP27 mutant infected cells. These results suggest that ICP27 functions post-transcriptionally (Smith et al., 1992).

There is evidence that ICP27 is involved in the post-transcriptional regulation of viral and cellular mRNA processing events (McLauchlan et al., 1992; Sandri-Goldin and Mendoza, 1992; Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994; Sandri-Goldin et al., 1995). In transient transfection assays, ICP27 enhances the expression of genes containing specific polyadenylation sequences and represses expression of genes containing introns 5′ or 3′ to the gene-coding sequences (Sandri-Goldin and Mendoza, 1992). Moreover, ICP27 is involved in inhibiting host cell splicing (Hardy and Sandri-Goldin, 1994) and is capable of altering the intranuclear distribution of splicing factors (Phelan et al., 1993; Sandri-Goldin et al., 1995). ICP27 may also regulate pre-mRNA polyadenylation. Increased processing efficiency at a late HSV-1 poly (A) site is dependent on ICP27 (McLauchlan et al., 1989; McLauchlan et al., 1992). The protein may increase the usage of weak poly (A) sites (McGregor et al., 1996).

ICP27 interacts with RNA *in vitro* (Brown et al., 1995; Ingram et al., 1996). Mears and Rice (1996) propose that ICP27 is an RGG box-type RNA binding protein and that the role of ICP27 in post-transcriptional regulation may be mediated by ICP27's RNA binding functions. ICP27 binds efficiently to poly (G) and weakly to poly (U) RNA homopolymers. In addition, this binding activity is dependent on and mediated by ICP27's RGG box. Further evidence to support the idea that ICP27 is an RGG box binding protein is the observation that ICP27 is methylated *in vivo*, a characteristic shared among many cellular RGG box proteins involved in mRNA and rRNA metabolism (Mears and Rice, 1996).

ICP27 coimmunoprecipitates with anti-Sm antiserum, demonstrating that ICP27 interacts, either indirectly or directly, with small nuclear ribonucleoprotein particles (snRNPs) (Sandri-Goldin and Hibbard, 1996). The snRNPs are essential components of the eukaryotic splicing apparatus (Luhrmann et al., 1990; Green, 1991; Moore et al., 1993). These results further support ICP27's proposed role in post-transcriptional mRNA processing.

ICP27 also appears to modulate the phosphorylation state of ICP4 (Rice and Knipe, 1988; Su and Knipe, 1989; McMahan and Schaffer, 1990). In an ICP27 mutant infection, ICP4 migrates slower on denaturing polyacrylamide gels than in a WT HSV-1 infection, indicating that ICP27 is involved, either directly or indirectly, in the phosphorylation of ICP4. (Rice and Knipe, 1988; Su and Knipe, 1989; McMahan and Schaffer, 1990). Interestingly, the various electrophoretic forms of ICP4 differ in their abilities to bind to specific DNA sequences (Michael et al., 1988; Papavassilious et al., 1991). These results suggest that ICP27 may indirectly regulate transcription by modulating ICP4's activity.

The regulatory functions of ICP27 may be mediated in part by interactions with other viral and cellular proteins. As described earlier in this chapter, ICP27 binds to ICP4, an interaction which may modulate the DNA binding activity of ICP4 by affecting ICP4's phosphorylation state (Panagiotidis et al., 1997).

d. Sequence-independent viral gene regulation

The activation of DE and L gene transcription may occur through sequence-independent mechanisms. Transcriptional control of viral genes may function at levels other than the 5' promoter regulatory regions, because viral genes or promoters outside the context of the viral genome are regulated differently from viral genes in their natural context. When a thymidine kinase chimeric gene, under the control of a promoter conferring true L (γ2) regulation, is placed into the cellular genome, the chimeric gene is expressed as a DE (β) gene product. Interestingly, the γ2-chimeric gene is regulated as a γ2 gene when inserted into a viral genome. These results suggest that there is differential regulation of viral genes depending on the environment in which they are placed (Silver and Roizman, 1985).

Moreover, cellular genes with their own promoters, when placed in the HSV-1 genome, are not transcriptionally repressed like their cellular counterparts, but are instead activated like viral genes (Smiley et al., 1987; Panning and Smiley, 1989; Smibert and Smiley, 1990). When the rabbit β-globin gene along with 1,200 bp of 5' sequence is stably integrated into the viral genome, the gene is expressed as a DE gene after infection of differentiated murine erythroleukemia (MEL) cells with the recombinant virus. The endogenous cellular β-globin gene is transcriptionally repressed, while the viral copy of the β-globin gene is transcriptionally activated (Smiley et al., 1987; Smibert and Smiley, 1990; Smiley et al., 1991). Similarly, when the α-globin gene is incorporated in the HSV-1 genome, the viral copy of the α-globin gene is expressed as a DE gene even in cells that do not express the endogenous cellular α-globin gene (Panning and Smiley, 1989).

These experiments suggest that the regulation of viral and cellular gene transcription may not involve differences in sequences between viral and cellular promoters. Sequence-independent mechanisms for the shift from cellular to viral transcription activation could include mobilization of cellular transcription factors, modification of RNAP II, differences in higher order chromatin packaging, or transport of DNA into specialized nuclear compartments (Smiley et al., 1991).

6. Viral kinases

a. ICP6

ICP6, encoded by the UL39 gene, is the large subunit of the HSV-1 ribonucleotide reductase (RR), an enzyme that is involved in creating precursors for DNA synthesis by reducing ribonucleotides to deoxyribonucleotides (Thelander and Reichard, 1979). ICP6 is a DE gene product whose expression is not dependent on ICP4 for its expression. Rather, ICP0 and VP16 are responsible for the transactivation of ICP6, as demonstrated in transient transfection assays (DeLuca and Schaffer, 1985; Desai al., 1993).

Although ICP6 plays a role in RR activity, ICP6 is able to function independently from the small subunit of RR. The N terminal domain of ICP6 has autophosphorylation and serine and threonine kinase activity *in vitro* (Chung et al., 1989; Paradis et al., 1991; Conner et al., 1992). Furthermore, ICP6 is phosphorylated *in vivo* (Bacchetti et al., 1984). Exogenous substrates phosphorylated *in vitro* by ICP6 include α-casein (Paradis et al., 1991), histone II-S (Paradis et al., 1991), the small subunit of RR (Peng et al., 1996), and immunoglobulin G (IgG) (Peng et al., 1996). ICP6 kinase activity is dependent on manganese cations (Peng et al., 1996).

b. US3

The US3 kinase, conserved only in α-herpesviruses (Baer et al., 1984; Davison and McGeoch, 1986; McGeoch and Davison, 1986), is nonessential for DNA replication in cell culture but is essential for neurovirulence in mice (Purves et al., 1987; Meignier et al., 1988). US3 is detected in the cytoplasm of infected cells and is present in virions (Purves et al., 1986b; Frame et al., 1987; Zhang et al., 1990). US3 phosphorylates the product of the UL34 gene, an essential virion nonglycosylated membrane protein (Purves et al., 1991; Purves et al., 1992), which in its unphosphorylated state associates with four novel viral phosphoproteins (Purves et al., 1992). ICP22 phosphorylation is also dependent on US3 (Purves et al., 1993), which may preferentially phosphorylate serine and threonine residues that are flanked by 4 to 6 arginine residues on the amino-terminal side of the target residue (Purves et al., 1986a).

c. UL13

UL13 (also called Vmw57), conserved in all α-, β-, and γ-herpesviruses (Davison and McGeoch, 1986; McGeoch, 1989; Smith and Smith, 1989), is an incoming component of the virion and is present in the tegument (Overton et al., 1992; Coulter et al., 1993). After infection, UL13 is localized to the nucleus (Cunningham et al., 1992) and is expressed late in the viral life cycle. UL13 is classified as a "late" (β) gene on the basis that it is present in infected cells at 6 hr post-infection and is reduced in protein and mRNA levels in the presence of DNA synthesis inhibitors (Costa et al., 1983; Overton et al., 1992). UL13 production commences between 3 and 6 hr post-infection and continues beyond 9 hr post-infection (Overton et al., 1992).

UL13 is a serine/threonine kinase (Smith and Smith, 1989; Cunningham et al., 1992; Overton et al., 1992) responsible for autophosphorylation (Cunningham et al., 1992; Overton et al., 1992) and the phosphorylation of the tegument protein VP22 (Coulter et al., 1993). UL13 is also required for normal ICP22 phosphorylation patterns, suggesting that ICP22 itself is a substrate of UL13 (Purves and Roizman, 1992). The phosphorylation of ICP22 by UL13 differs from that by US3 (Purves et al., 1993) and is mediated late in infection (Purves et al., 1993).

UL13 is nonessential for virus growth in cell culture but viral replication is impaired in some cell lines (rabbit skin cells, BHK, HEL cells) in the absence of the UL13. Interestingly, ICP22 mutants show the same growth impairments as UL13 mutants in similar cell lines (Purves et al., 1993; Overton et al., 1994). In a restrictive infection, UL13 mutants show reduced levels of ICP0 and several late viral proteins (Purves et al., 1993). A host factor may be capable of substituting for UL13 function in permissive cells.

It appears that UL13 may be required for the virion host shutoff effect (Overton et al., 1994), an event that will be described below. The inability of UL13 to induce virion host shutoff is not due to a reduction in the vhs protein, another protein which is required for virion host shutoff (Overton et al., 1994). It is currently unknown whether UL13 phosphorylates the vhs protein or how UL13 induces virion host shutoff.

7. Host shut off

After HSV-1 infection, there is a general repression of host cell metabolic processes. Host DNA synthesis is shut off (Kwong et al., 1988). Host protein (Kwong and Frenkel, 1987; Read and Frenkel, 1983) and ribosomal RNA (Wagner and Roizman, 1969) synthesis are reduced. The glycosylation of host proteins is also inhibited (Spear et al., 1970). There is also a general down-regulation of cellular gene transcription (Godowski and Knipe, 1986; Weinheimer and McKnight, 1987; Kemp and Latchman, 1988a; Smibert and Smiley, 1990; Spencer et al., 1997), an inhibition of mRNA splicing (Hardy and Sandri-Goldin, 1994), and the increased degradation of cellular mRNAs (reviewed in Smiley et al., 1991).

a. Virion host shutoff (Vhs)

The virion host shutoff (*vhs*) function is mediated by the vhs protein, which is encoded by the UL41 gene (Kwong et al., 1988) and is packaged in the tegument of the virion (Smibert and Smiley, 1990). The 58 kDa vhs protein is post-translationally modified by phosphorylation (McGeoch et al., 1988; Smibert et al., 1992), and has limited homology to a small segment of poly (A) binding protein (Sorenson et al., 1991). The protein likely contains a nuclear localization or retention signal as it localizes to the nucleus when expressed in transfected cells (Friedman et al., 1988).

The *vhs* function enters the host cell as part of the virion and acts in the absence of viral gene expression. It mediates the shutoff of host protein synthesis 5 to 10 fold by destabilizing and degrading most cellular mRNAs (Schek and Bachenheimer, 1985; Kwong and Frenkel, 1987; Strom and Frenkel, 1987; Kwong et al., 1988). The *vhs* gene product also destabilizes and degrades viral IE, DE, and L mRNAs (Kwong and Frenkel, 1987; Oroskar and Read, 1987; Strom and Frenkel, 1987; Fenwick and Owen, 1988; Kwong et al., 1988). Infection of cells with a *vhs* mutant virus results in inefficient host shutoff and prolonged IE and DE protein synthesis. In *vhs* mutant infected cells, the half-lives of both host and viral mRNAs are lengthened (Kwong and Frenkel, 1987).

The increased degradation of both viral and cellular mRNAs may provide distinct advantages to the cell. Degradation of host mRNA may allow for preferential translation of viral mRNAs synthesized during infection. Viral mRNAs are synthesized in such large quantities that *vhs* mediated degradation does not entirely eliminate them from the infected cell. However, host transcription is repressed after virus infection and host mRNAs are efficiently reduced by *vhs* degradation. Also, the high rate of turnover of viral mRNAs may facilitate the transition of transcription from one viral gene class to another (Kwong and Frenkel, 1987). It is of interest that the *vhs* function specifically affects mRNAs. Host rRNAs (Zelus et al., 1996) and tRNAs are unaffected (Oroskar and Read, 1987).

The precise mechanism of *vhs* function remains to be determined. *Vhs* function may be mediated by interactions between VP16 and the vhs protein (Smibert et al., 1994). The vhs protein interacts with one or more regions of VP16 that are required for promoter recognition (Smibert et al., 1994). These interactions may be instrumental in modulating the activity of the vhs protein during infection (Smibert et al., 1994). Results from experiments performed by Lam et al. (1996) demonstrate that VP16 dampens vhs activity at intermediate to late times post-infection (5 to 11 hr), allowing viral mRNAs to persist in infected cells (Lam et al., 1996).

Jones et al. (1995) demonstrate that the vhs protein is capable of functioning when expressed in isolation and in the absence of other viral proteins. However, in infected cells, at least one other virion protein, the UL13 viral kinase, appears to be required for efficient *vhs* function. UL13 mutant infected cells display a vhs mutant phenotype despite having WT levels of vhs protein. The mechanism by which UL13 induces *vhs* activity is currently undefined (Overton et al., 1994). It is possible that UL13 modulates vhs-VP16 complexes by phosphorylating the vhs protein or VP16 early in infection (Jones et al., 1995).

Although the vhs protein lacks sequence homology to any known RNases, it may be capable of functioning as a messenger ribonuclease (mRNase). Crude extracts from virions or *in vitro* translated vhs protein is capable of degrading various RNA substrates (Zelus et al., 1996). Therefore, the vhs protein alone or in conjunction with another protein may directly degrade mRNA (Zelus et al., 1996).

b. Cellular transcriptional repression

After HSV-1 infection, there is a general reduction in host RNA synthesis (Hay et al., 1966; Wagner and Roizman, 1969). Transcription by all three nuclear RNA polymerases generally declines to less than 50% of uninfected cells after 4 hr post-infection (Preston and Newton, 1976). There are some reports in the literature of increased RNAP III transcription of endogenous human Alu elements following HSV-1 infection (Jang and Latchman, 1989; Panning and Smiley, 1994).

Nuclear run-on transcription assays have shown that HSV-1 infection brings a 40% decrease in histone H3 and actin gene transcription at 4 hr post-infection (Mayman and Nishioka, 1985). Furthermore, a 95% decrease in β-globin gene transcription occurs in differentiated MEL cells at 5 hr post-infection (Smibert and Smiley, 1990). Transcription of some unidentified cDNAs is also reduced after 5 hr post-infection (Kemp and Latchman, 1988a). Lastly, recent studies show a repression of c-myc, c-fos, γ-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and histone H2b gene transcription after HSV-1 infection (Spencer et al., 1997).

Not all cellular gene transcription is repressed. Transcription of ubiquitin B, interleukin 6, α-globin and several unidentified genes, may increase after HSV-1 infection (Kanangat et al., 1966; Kemp and Latchman, 1988a; Kemp and Latchman, 1988b; Preston, 1990; Cheung et al., 1997).

ICP27 is required for the repression of cellular gene expression at the levels of protein synthesis and mRNA accumulation (Sacks et al., 1985; Hardwicke and Sandri-Goldin, 1994). It has been thought that ICP27's effects on host gene expression are the result of its inhibitory effects on mRNA splicing. However, recent studies suggest that ICP27's effects on cellular gene expression may also occur at the transcriptional level (Spencer et al., 1997). Nuclear run-on experiments show that multiple IE gene products contribute to the repression of host RNAP II transcription. Full repression of cellular gene expression appears to require the presence of ICP27 (Spencer et al., 1997).

8. Latency

HSV-1 (neurotropic) latency involves the restriction of lytic gene expression and the sustained presence of the viral genome in neurons. The process of latency involves three phases: establishment, maintenance and reactivation. After a primary lytic infection, some virus progeny are released from the epithelial cells to infect adjacent sensory nerve endings. Once HSV-1 has entered the neurons, a short period of productive infection occurs and then latency is established, a state where viral gene transcription is generally quiescent. No viral proteins are required for latency. In the neuron, the viral genome is episomal and is not integrated into the host genome (Wagner, 1991)

Reactivation occurs in response to stresses such as physical injury, environmental factors (sunburn), and disease (fever). Reactivation involves a small amount of viral replication in the neurons. The virus then travels through the sensory ganglia to infect peripheral epithelial cells at or near the primary site of infection. Productive lytic infection then occurs in the epithelial cells. Characteristic cold sores or lesions caused by HSV-1 often reoccur due to alternating lytic and latent cycles of infection (Wagner, 1991).

The mechanisms by which reactivation occur are not well understood. Only one major RNA transcript is detected in neurons during latency. This RNA, termed the HSV-1 latency-associated transcript (LAT), is approximately 2,000 nucleotides in length, is present only in the nucleus and is not polyadenylated. The LAT gene is transcribed in the opposite orientation to the ICP0 gene and overlaps the 3′ end of the ICP0 gene. LAT gene expression does not appear to be required for the establishment of latency. However, reactivation appears to require the transcription of the LAT gene (Wagner, 1991) and ICP0 activity (Leib et al., 1989; Cai et al., 1993).

F. Thesis objective

The question of how HSV-1 activates viral transcription while suppressing cellular transcription is important in understanding both viral regulatory mechanisms and the fundamental mechanisms of RNAP II transcription regulation. The profound transcriptional shifts that occur following HSV-1 infection appear to be unrelated to the DNA sequences of cellular and viral promoters. It has been proposed that sequence-independent mechanisms, including the mobilization of cellular transcription factors, modification of RNAP II, differences in higher order chromatin packaging, or transport of DNA into specialized nuclear compartments, may lead to the global shifts in transcription patterns that follow HSV-1 infection (Smiley et al., 1991).

In order to address the mechanisms of virus-induced redirection of RNAP II transcription, I have examined modifications to the RNAP II transcription machinery, the transcription changes that accompany HSV-1 infection, and the viral regulatory genes that control these processes. My specific goals were three-fold:

- To characterize modifications to the RNAP II basal transcription apparatus following HSV-1 infection.
- To determine how the virus brings about these modifications.
- To understand how these modifications may contribute to RNAP II transcription changes during HSV-1 infection.

My work has provided insight into several important aspects of transcription regulation during virus infection. I have shown that:

- RNA polymerase II is aberrantly phosphorylated and localized to viral replication compartments following herpes simplex virus infection.
- The IE protein ICP22 is required for viral modification of host RNA polymerase II and establishment of the normal viral transcription program.
- RNAP II modifications and efficient viral transcription are dependent on the presence of the virion kinase UL13.
- DNA-PK protein kinase activity and its catalytic subunit are attenuated by the HSV-1 transactivator ICP0.
- ICP22 interacts with ICP4.

The research reported in this thesis provides the basis for an enriched model of how RNAP II transcription is commandeered by viruses and redirected to specific sets of genes.

Table 1.1 Classification of the herpesviridae family

Subfamily	Examples	Host
Alpha(α)-herpesviruses	herpes simplex virus type 1 (HSV-1)	human
	herpes simplex virus type 2 (HSV-2)	human
	pseudorabies virus (PRV)	swine
	monkey B virus	monkey
	equine herpesvirus type 1 (EHV-1)	horse
Beta(β)-herpesviruses	human cytomegalovirus (HCMV)	human
	human herpesvirus type 6 (HHV-6)	human
	simian cytomegalovirus	monkey
Gamma(γ)-herpesviruses	Epstein-Barr virus (EBV)	human
	herpesvirus saimiri	monkey
	Marek's disease herpesvirus	chicken
	Kaposi's sarcoma-associated herpesvirus (KSHV) (human herpes virus 8 (HHV-8))	human

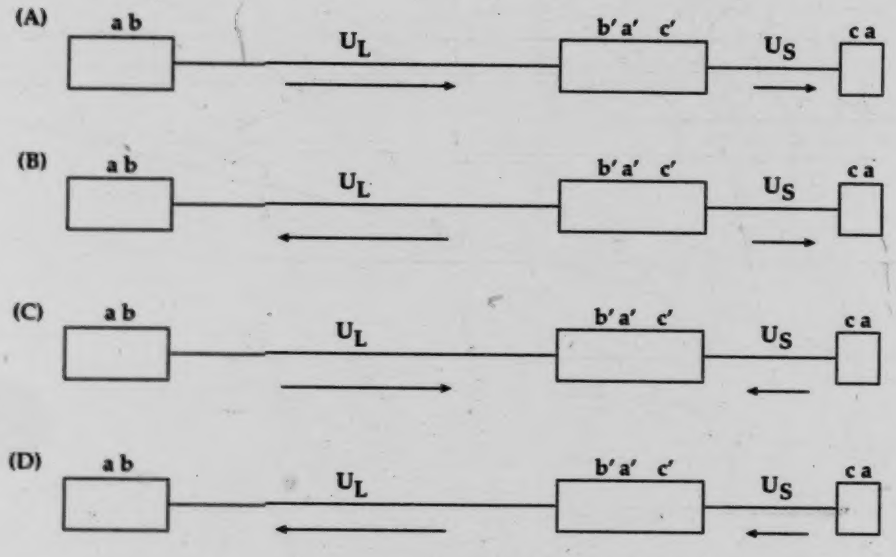


Figure 1.1. Schematic representation of the HSV-1 genome. The long (U_L) and short (U_S) regions of DNA are covalently linked and are denoted by the thin lines. The U_L and U_S sequences are flanked by inverted terminal repeats, represented by the boxes. The repeats of the U_L region are ab and b'a', while the repeats of the U_S region are a'c' and ca. The HSV-1 genome isomerizes during viral DNA replication, resulting in a single virion containing one of four configurations (A to D). The U_L and U_S regions invert relative to one another. Equal amounts of viral progeny are produced containing each isoform (Roizman and Sears, 1990).

Figure 1.2. Simplified layout of the HSV-1 genome. The HSV-1 genome is presented on four lines. The U_L and U_S regions of the DNA are denoted by the thin lines, whereas the terminal repeats are shown as boxes. The scale represents fractional map units. The sizes, orientations, and positions of functional open reading frames of various HSV-1 genes (ICP0, ICP4, ICP6, ICP8, ICP22, ICP27, UL13, US3, vhs and VP16) are roughly estimated and drawn as arrows (McGeoch et al., 1988).

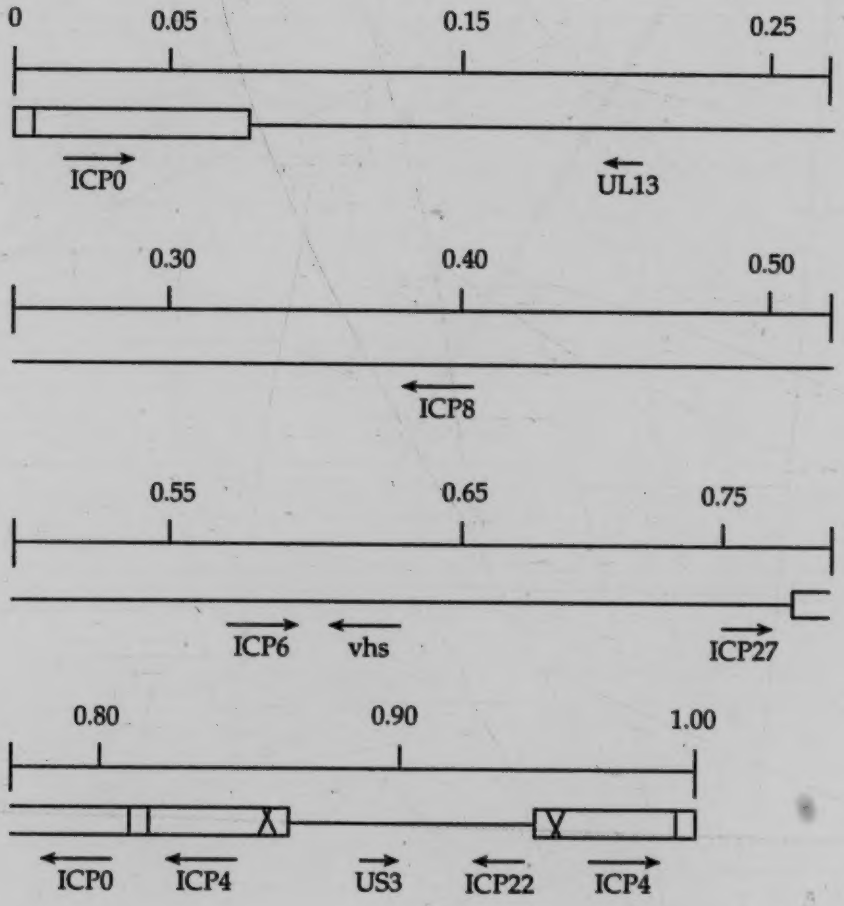
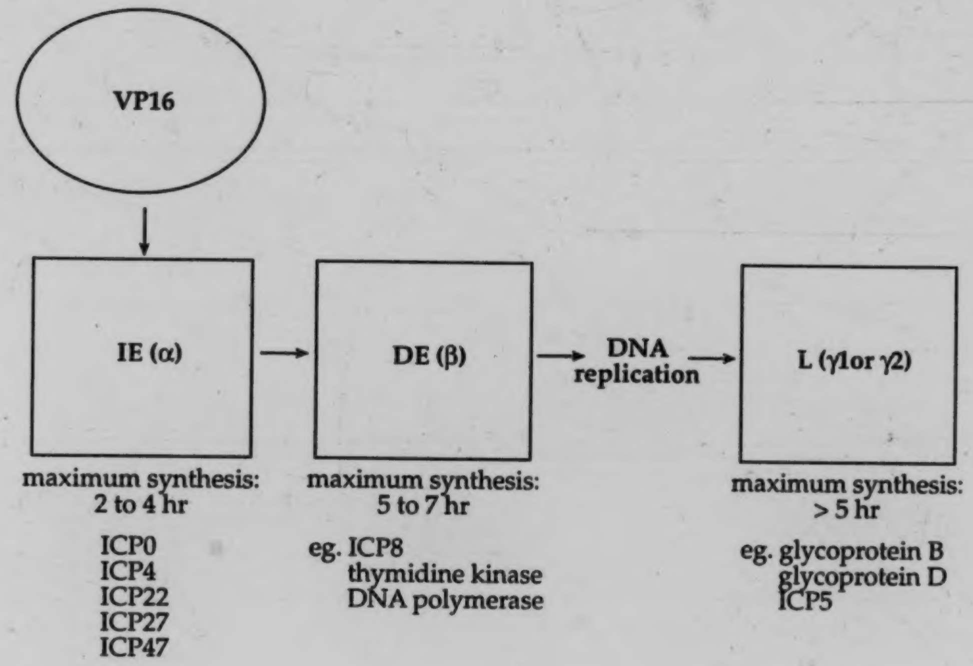

0
0.05
0.15
0.25
ICP0
UL13
0.30
0.40
0.50
ICP8
0.55
0.65
0.75
ICP6
vhs
ICP27
0.80
0.90
1.00
ICP0
ICP4
US3
ICP22
ICP4

Figure 1.3. HSV-1 temporal gene expression cascade. During lytic infection, the HSV-1 genes are expressed in a sequential order and are regulated primarily at the transcription level. VP16, a component of the virion particle enters the cell after infection. VP16 in complex with host cell factors stimulates transcription of IE (α) genes, which include ICP0, ICP4, ICP22, ICP27, and ICP47. Maximum synthesis of IE proteins occurs between 2 and 4 hr post-infection. The first four IE proteins are required for the subsequent expression of DE (β) and L (γ) genes. DE gene expression is dependent on the presence of ICP4. DE proteins are mainly those involved in nucleic acid metabolism and viral DNA replication. These proteins DE include ICP8, the viral thymidine kinase and the viral DNA polymerase and are expressed maximally between 5 and 7 hr post-infection. L gene products are expressed after DE genes and encode structural proteins, including glycoprotein B, glycoprotein D and ICP5. The L genes are dependent on the presence of ICP4 and ICP27 for their expression and are also partially (γ1) or wholly (γ2) dependent on viral DNA replication. L proteins are maximally synthesized after 5 hr post-infection (Roizman and Sears, 1990).


VP16
IE (α)
DE (β)
DNA replication
L (γ1or γ2)
maximum synthesis: 2 to 4 hr
ICP0
ICP4
ICP22
ICP27
ICP47
maximum synthesis: 5 to 7 hr
eg. ICP8
thymidine kinase
DNA polymerase
maximum synthesis: > 5 hr
eg. glycoprotein B
glycoprotein D
ICP5

CHAPTER TWO. MATERIALS AND METHODS

A. Cells, viruses, and infections

1. Cells

African green monkey kidney (Vero), human epithelioid cervical carcinoma (HeLa S3 or HeLa CCL2.2), and human embryonic lung (HEL 299) cells were used for infections. All cell lines were obtained from the American Type Culture Collection, Rockville, Md. Vero, HeLa CCL2.2, and HEL 299 cells were grown as monolayer cultures. HeLa S3 cells were grown as monolayer or suspension cultures. Vero and HEL 299 cells were propagated in Dulbecco modified Eagle's medium (D-MEM) containing 5% fetal calf serum, 1% penicillin, and 1% streptomycin. HeLa S3 and HeLa CCL2.2 cells were propagated in D-MEM containing 10% heat-inactivated newborn calf serum, 1% penicillin, and 1% streptomycin. All tissue culture reagents were purchased from Gibco BRL, Gaithersburg, Md.

a. Stably transfected HeLa S3 F-ICP22 cells

HeLa S3 cells were stably transfected with the pUHD 15-1 plasmid, which contains the tTA gene encoding the tetracycline (tet)-VP16 fusion protein (Gossen and Bujard, 1992). The tet-VP16 activator protein is constitutively expressed from the human cytomegalovirus (HCMV) promoter/enhancer. The stably transfected tet-VP16 expressing cell line was termed cell line 33.

In order to design a FLAG-tagged ICP22 gene, a 30 base pair double-stranded synthetic oligonucleotide encoding the 8 amino acid FLAG sequence (DYKDDDDK) plus flanking sequences, was inserted, in frame, into the cloned HSV-1 ICP22 gene, replacing amino acids 6 to 9. The FLAG-tagged ICP22 gene (F-ICP22) was then cloned into the pUHD 10-3 plasmid (Gossen and Bujard, 1992). This places the F-ICP22 gene under control of the HCMV minimal promoter and 7 copies of the tet repressor binding site. The F-ICP22/HCMV construct was stably transfected into cell line 33, which expresses tet-VP16 protein. Cloning and stable transfections were performed by Alison Kilvert, University of Alberta, Edmonton, Alberta.

The stably transfected cell lines were propagated and maintained in D-MEM containing newborn calf serum, 1% penicillin, 1% streptomycin, 200 µg/ml hygromycin, 1 mg/ml geneticin, and 1 µg/ml tetracycline. Cell line 33 was grown in similar media except that no hygromycin or tetracycline was used.

2. Viruses

a. WT and mutant HSV-1

Wild-type HSV-1 strain KOS1.1 (Hughes and Munyon, 1975) was originally obtained from Dr. Myron Levine, University of Michigan, Ann Arbor. The ICP4 mutant virus *d*120 was provided by Dr. Neal DeLuca, University of Pittsburgh School of Medicine, Pittsburgh, PA. The *d*120 virus contains a deletion in the ICP4 gene, where only the first 171 amino acids of the 1298 amino acid protein are expressed (DeLuca et al., 1985; DeLuca and Schaffer, 1988). The *d*120 virus was grown and titered on E5 cells, which contain the gene encoding ICP4 stably integrated within its genome (DeLuca et al., 1985). The ICP27 mutant virus *d*27-1, containing a deletion in the gene encoding ICP27, was grown and titered on V27 cells (Rice and Knipe, 1990). V27 cells contain the ICP27 gene stably integrated within its genome (Rice and Knipe, 1990). The ICP0 mutant virus *n*212, the ICP22 mutant virus 22/*n*199, and the marker rescued derivative of 22/*n*199, 22/*n*199R, were provided by Dr. Priscilla Schaffer, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts. The *n*212 virus, containing a nonsense mutation in both copies of the ICP0 gene (Cai et al., 1993), was grown and titered in 0-28 cells, which are Vero cells that contain multiple copies of the ICP0 gene incorporated into its genome (Sacks and Schaffer, 1987). The 22/*n*199 virus contains a nonsense mutation in the ICP22 gene. To construct the 22/*n*199 virus, a 16 bp oligonucleotide linker sequence containing stop codons in all three reading frames was inserted in the single *Pvu* II site in the ICP22 coding region. The ICP22 gene of 22/*n*199 encodes a truncated ICP22 protein consisting of the N-terminal 199 amino acids of the 420 residue ICP22 protein. Both 22/*n*199 and 22/*n*199R viruses were grown and titered on Vero cells (Astor et al., Unpublished data). The ICP6 mutant virus ICP6 was obtained from Dr. Sandy Weller, University of Connecticut Health Center, Farmington, CT. The ICP6 mutant virus ICP6 lacks 90% of the ICP6 coding sequence and only contains the last 491 bp of the ICP6 gene carboxy-terminal coding region (Goldstein and Weller, 1988). The ICP6 virus was grown and titered on Vero cells at 34°C (Goldstein and Weller, 1988). The ICP8 mutant virus *d*101 contains an internal deletion in the ICP8 gene and was grown and titered on S2 cells, which are Vero cells that contain 1 to 10 copies of the ICP8 gene within its genome (Gao and Knipe, 1989).

The *d*22 virus, constructed by Dr. Stephen Rice and Vivian Leong (Lam), University of Alberta, Edmonton, Alberta, contains a complete deletion of the ICP22 gene and was grown and titered on Vero cells. The *d*22 virus was prepared by cotransfecting Vero cells with the pUC-NS-lac Z (8.5 kb) plasmid and WT HSV-1 DNA. pUC-NS-lacZ contains the lac Z gene (encodes glycoside hydrolase, β-D-galactosidase) with flanking non-protein-coding ICP22 sequences (DNA sequences between *Age* I (129) to *Bgl* II (441) and between *Eag* I (1675) to *Age* I (3364) in the ICP22 gene). The *d*22 virus was isolated and plaque purified.

The UL13 mutant virus *d*UL13, constructed by Dr. Stephen Rice, contains a deletion in the UL13 gene and was grown and titered on Vero cells. The *d*UL13 mutant encodes sequences only for the first 154 amino acids of the 518 amino acid UL13 protein and was constructed by marker transfer with WT HSV-1 DNA and the pBgl OZ-alpha plasmid, which contains the lac Z gene with flanking N terminal UL13 protein-coding sequences and non-protein-coding 3′ UL13 sequences. The *d*UL13 virus was isolated and plaque purified.

The F22 virus, prepared by Dr. Stephen Rice and Vivian Leong (Lam), was grown and titered on Vero cells. F22 is a recombinant HSV-1 virus containing an 8 amino acid FLAG epitope DYKDDDDK inserted 8 amino acids from the amino terminus of the ICP22 protein. ICP22 containing the FLAG epitope sequences was designated as F-ICP22. The F22 virus was isolated and plaque purified.

b. Ultraviolet (UV)-inactivated HSV-1

A stock of WT HSV-1 KOS1.1 was ultraviolet (UV) irradiated by Dr. Stephen Rice and Vivian Leong (Lam) using the following procedure. The virus stock was thawed and 2 ml aliquots were placed in 10 cm diameter petri dishes. The open petri dishes were then irradiated with 254 nm light in a StrataLinker UV Crosslinker 2400 apparatus from Stratagene (~4,000 $\mu W/cm^2$) for various periods of time. The aliquots were then dispensed and refrozen, and titers were determined by plaque assay on Vero cells. Ten min of UV irradiation reduced the titer of the stock by 4 to 5 orders of magnitude compared with that of a mock-irradiated sample.

The 10 min UV-irradiated stock was subsequently tested for the expression of immediate-early (IE) proteins after infection. When UV-irradiated virus was used to infect Vero cells, less than 0.1% of the cells were positive by immunofluorescence for the IE protein ICP27 at 5 hr post-infection. Infection by the mock-irradiated stocks resulted in > 95% positive ICP27 immunofluorescence at 5 hr post-infection. Infections (section A.3) and immunofluorescence (section C) are described in further detail in this chapter.

c. Other DNA viruses

Wild-type HSV-2 (strain G) was obtained from the American Type Culture Collection. Wild-type vaccinia virus (strain WR, Wyeth Reserve) was obtained from Dr. Grant McFadden, University of Alberta, Edmonton, Alberta.

3. Infections

Cells were infected with virus at a multiplicity of infection (MOI) of 10 PFU per cell in phosphate-buffered saline (PBS) (137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM sodium phosphate monobasic heptahydrate, and 1.4 mM potassium dihydrogen phosphate) containing 0.1% glucose and 1% heat-inactivated newborn calf serum. In monolayer cultures, the virus inoculum was allowed to adsorb to the cells for 1 hr at 37°C. The inoculum was then replaced with 199 medium containing 2% newborn calf serum, 1% penicillin, and 1% streptomycin. The cultures were then incubated at 37°C.

The 10 min UV-irradiated stock was used to infect cells whereas the mock-irradiated stock served as the WT control. All infections with the UV irradiated stocks were done using the stock's preirradiated titer to obtain a MOI of 10 PFU per cell.

Infections with vaccinia virus (VV) were performed in logarithmic HeLa S3 cells grown as a suspension culture. The cells were pelleted by low-speed centrifugation and resuspended in 0.1 volume of D-MEM containing 10% newborn calf serum, 1% penicillin, and 1% streptomycin. The cell culture was infected at a MOI of 10 with freshly sonicated VV. The infected cultures were incubated at 37°C. After 1 hr, the cells were rediluted back to the original volume, and the culture was incubated at 37°C.

B. Antibodies

Two mouse monoclonal antibodies recognizing the large subunit of RNAP II were used: 8WG16 (Thompson et al., 1989; Thompson et al., 1990) and ARNA3 (Krämer et al., 1980; Krämer and Bautz, 1981). 8WG16 was obtained from Dr. Nancy Thompson, University of Wisconsin, Madison, WI, and ARNA3 was purchased from Cymbus Bioscience Ltd., Southhampton, United Kingdom. 8WG16, an IgG_{2a} antibody, recognizes epitopes on the CTD of the large subunit (Thompson et al., 1989; Thompson et al., 1990) and reacts with all phosphorylation variants of the large subunit of RNAP II except for hyperphosphorylated forms (IIo). ARNA3, an IgG_1 antibody, recognizes epitopes on the body of the large subunit and reacts with all forms of the large subunit of RNAP II. For Western blotting, 8WG16 was used at a dilution of 1:2,000 (6 µg/ml), and ARNA3 was used at a dilution of 1:50 (1 µg/ml) in PBS containing 0.1% Tween 20 (PBS-T) and 0.02% sodium dodecyl sulfate (SDS). For immunofluorescence, 8WG16 was used at a dilution of 1:200 (60 µg/ml), and ARNA3 was used at a dilution of 1:6 (8.3 µg/ml) in PBS.

TBP18, a mouse monoclonal antibody directed against TBP, was obtained from Dr. Nancy Thompson, University of Wisconsin, Madison, WI. In Western blotting, TBP18 was used at a dilution of 1:750 (0.16 μg/ml) in PBS-T with 0.05% SDS. In immunofluorescence studies, TBP18 was used at a dilution of 1:200 (0.6 μg/ml) in PBS. The α–TFIIB, α–p56E, and α–RAP74 antibodies were obtained from Dr. Jack Greenblatt, University of Toronto. The α–TFIIB antibody is an affinity purified rabbit polyclonal antibody directed against human TFIIB and was used at a 1:3,000 dilution in PBS-T with 0.05% SDS in Western blotting. The α–p56E antibody is an affinity purified rabbit polyclonal antibody directed against the 56 kDa subunit of human TFIIE. The epitope for α–p56E is between amino acids 423 to 439 of the carboxy-terminus of p56. The α–p56E antibody was used at a 1:500 dilution in PBS-T with 0.02% SDS in Western blotting. The α–RAP74 antibody is a rabbit polyclonal antibody directed against the RAP74 subunit of human TFIIF. The epitope for α–RAP74 is between amino acids 498 to 515 on the carboxy-terminus of RAP74. The α–RAP74 antibody was used at a 1:300 dilution in PBS-T with 0.02% SDS in Western blotting. The MO1.1 antibody is a mouse monoclonal antibody (Tassan et al., 1994) and was obtained from Dr. Erich Nigg, University of Geneva, Geneva, Switzerland. MO1.1 was used at a dilution of 1:10,000 in PBS-T with 0.02% SDS in Western blotting.

Antibodies directed against other cellular proteins were also used. The PKC antibody is a rabbit polyclonal antibody directed against protein kinase C and was used at a 1:500 (0.006 μg/ml) concentration in Tris-buffered saline (TBS), 0.1% Tween 20, 5% bovine albumin serum (BSA), and 0.02% sodium azide in Western blotting. The phosphatase-1 antibody is a rabbit polyclonal antibody directed against phosphatase-1 and was used in Western blotting at a concentration of 1:500 in TBS, 0.1% Tween 20, 5% BSA, and 0.02% sodium azide. The PKC and phosphatase-1 antibodies were provided by Dr. Jim Stone, University of Alberta, Edmonton, Alberta and originally from Upstate Biotechnology Inc., Lake Placid, NY.

DPK1, a rabbit polyclonal antibody raised to amino acids 2018 to 2136 of p350/DNA-PKcs (Lees-Miller et al., 1995; Chan et al., 1996), was obtained from Dr. Susan Lees-Miller, University of Calgary, Calgary, Alberta. DPK1 was used in Western blotting at a concentration of 1:2,500 in PBS-T with 0.02% SDS and in immunofluorescence at a concentration of 1:100 in PBS containing 1% BSA. Also obtained from Dr. Susan Lees-Miller was Ku24.2, which is a mouse polyclonal antibody that recognizes both the p70 and p80 subunits of Ku (Chan and Lees-Miller, 1996). Ku24.2 was used at a dilution of 1:2,500 in PBS-T with 0.02% SDS in Western blotting and at a dilution of 1:100 in PBS in immunofluorescence.

Antibodies recognizing specific HSV-1 proteins were as follows: H1112, a mouse monoclonal antibody directed against ICP0, was used at a dilution of 1:1,000 in PBS-T with 0.02% SDS in Western blotting. Two mouse monoclonal antibodies recognizing the IE protein ICP4 were used: H1101 and H1114. H1101, an IgG antibody, recognizes a region near the amino terminus of ICP4 and was used at a 1:400 dilution in PBS-T with 0.02% SDS in Western blot analyses. H1114, an IgG_{2a} antibody, recognizes regions of ICP4 following amino acid 171 (Leong [Lam] and Mears, 1996, Personal communication) and was used at a 1:2,700 dilution in PBS-T with 0.02% SDS in Western blotting. H1113, a mouse monoclonal antibody directed against ICP27 (between amino acid 109 and 138) (Leong [Lam] and Mears, 1996, Personal communication), was used at a concentration of 1:500 in PBS-T containing 0.02% SDS in Western blotting and at a concentration of 1:1,000 in PBS in immunofluorescence. H1115, a mouse monoclonal antibody directed against ICP8, was used at a concentration of 1:300 in PBS-T with 0.02% SDS in Western blotting. H1115 was used in immunofluorescence at a concentration of 1:1,000 in PBS. The 3-83 antibody, a rabbit polyclonal antibody, specifically recognizes ICP8 (Knipe et al., 1987) and was used in immunofluorescence studies at a concentration of 1:200 in PBS. All viral antibodies were purchased for the Goodwin Institute, Plantation, Fla. with the exception of 3-83, which was obtained from Dr. David Knipe, Harvard Medical School, Boston, Massachusetts.

M2 is a mouse monoclonal antibody that recognizes the FLAG epitope and was purchased from Eastman Kodak Company, New Haven, CT. M2, an IgG_1 antibody, was used to detect F-ICP22 protein at a concentration of 1.1 μg/ml PBS-T with 0.02% SDS in Western blotting. M2 was used to detect F-ICP22 at a dilution of 1:1,000 (3.5 μg/ml) in immunofluorescence.

Secondary antibodies used for Western blot analyses were affinity purified horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibodies. Secondary antibodies used in Western blotting were diluted to a concentration of 1:100,000 in PBS-T with varying percentages of SDS in the incubation and wash buffers. The secondary antibodies used for immunofluorescence studies were rhodamine-conjugated goat anti-mouse immunoglobulin G (GAMR) and dichlorotriazinyl amino fluorescein donkey anti-rabbit immunoglobulin G (heavy and light chains) (DARF). GAMR and DARF were used at a concentration of 1:200 in PBS. Also used in immunofluorescence studies were α-rabbit biotinylated antibody, α-mouse biotinylated antibody, α-streptavidin fluorescein, and α-streptavidin Texas Red, which were used at dilutions of 1:100 in PBS and 1% BSA. All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc., Mississauga, Ontario.

C. Indirect immunofluorescence

Indirect immunofluorescence was performed as previously described (Quinlan et al., 1984). Vero, HeLa (S3 or CCL2.2), or HEL 299 cells were grown to confluence on coverslips and mock infected or infected with WT or mutant HSV-1 for various times. In some experiments, HeLa CCL2.2 cells were grown and infected specifically because these cells form better monolayers than HeLa S3 cells which tend to overlap during growth. The cells were fixed for 10 min in 3.7% formaldehyde in PBS and then permeabilized for 2 min in cold (-20°C) acetone. The coverslips were rinsed in PBS and incubated with one (single immunofluorescence) or two (double immunofluorescence) primary antibodies at different concentrations for 30 min at 37°C. The coverslips were rinsed in PBS and then incubated with one (single immunofluorescence) or two (double immunofluorescence) chromophore conjugated secondary antibodies, GAMR and/or DARF, at a dilution of 1:200 for 30 min at 37°C. The coverslips were rinsed in PBS and mounted onto slides with glycerol gelatin containing 4 mg/ml N-propyl gallate (NPG). NPG prevents fading of the fluorescein signal. Both glycerol gelatin and NPG were purchased from Sigma Chemical Company, Mississauga, Ontario.

An alternate immunofluorescence method was used to detect p350/DNA-PKcs and Ku. HeLa CCL2.2 cells were grown to confluence on coverslips and mock infected or infected with WT virus for 5 hr. The cells were fixed and permeabilized in one step by incubating the coverslips in 3.7% formaldehyde, 0.5% Triton X-100, and 0.05% Tween-20 in PBS for 10 min. The coverslips were incubated with either rabbit polyclonal DPK1 or Ku24.2 primary antibodies for 30 min at 37°C. The coverslips were then washed in PBS containing 0.05% Tween 20 for 10 min at room temperature and then incubated with α-rabbit biotinylated or α-mouse biotinylated secondary antibody for 30 min at 37°C. The cells were washed with PBS containing 0.05% Tween 20 for 10 min at room temperature and incubated with α-streptavidin fluorescein or α-streptavidin Texas Red for 30 min at 37°C. The coverslips were washed in PBS containing 0.05% Tween 20 for 10 min at room temperature, rinsed in distilled water, and mounted onto slides with gelatin containing NPG.

The cells were visualized at a 63x and 100x (Plan Neofluor objective lens) magnification with a Zeiss Axioskop 20 fluorescence microscope. Photography of the results was performed using Kodak Ektachrome 400 color slide film and Kodak Tmax 400 black and white print film.

D. Western blot analysis

1. Protein extract preparation

Whole cell extracts were prepared from 3×10^6 to 4×10^6 cells grown to confluence in 25 cm^2 flasks. Cells were either mock infected or infected with WT or mutant virus. At various post-infection times, the cells were scraped and rinsed in PBS containing protease inhibitors N-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (50 µg/ml) and phenylmethylsulfonyl fluoride (PMSF) (25 µg/ml) from Sigma Chemical Company. In some experiments, the following phosphatase inhibitors were included in the scraping buffer: 1 mM sodium orthovanadate, 25 mM sodium fluoride, 50 mM tetrasodium phosphate, 50 mM sodium pyrophosphate, 20 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). In some experiments, the cells were counted with a haemocytometer. The cells were pelleted by low speed centrifugation and then lysed in 250 µl of Laemmli sample buffer (58.7 mM Tris-hydrochloric acid (HCl) pH 6.9, 1.88% SDS, 18.8% glycerol, 0.5% β-mercaptoethanol, and 0.002% bromophenol blue) (Laemmli, 1970) per 10^6 cells.

In some experiments, the cells were not harvested by scraping but instead treated with trypsin. In these experiments, the trypsinized cells were collected and diluted in an equal volume of D-MEM. The cells were collected by low speed centrifugation and then washed in PBS containing a mixture of protease and/or phosphatase inhibitors as described above. The cells were counted, pelleted and resuspended in 250 µl of Laemmli sample buffer per 10^6 cells.

Vaccinia virus (VV) infected HeLa S3 extracts were prepared from a suspension culture. HeLa S3 cells were infected with VV. At different post-infection times, aliquots of infected cells were removed from the suspension culture and harvested by low-speed centrifugation at 1,000 rpm for 5 min at 4°C. The pellet was washed in PBS containing the protease and phosphatase inhibitors as described above. The cells were pelleted by centrifugation at 1,000 rpm for 5 min at 4°C and then resuspended in 250 µl of Laemmli sample buffer per 10^6 cells.

Whole cell extracts were also provided by other laboratories for Western blot analyses. Varicella-zoster virus (VZV) infected human melanoma (MeWos) cell extracts were obtained from Dr. Bill Ruyechan and Dr. David Stevenson, University of Buffalo, Buffalo, NY. Human cytomegalovirus (HCMV) infected HEL 299 cell extracts were obtained from Dr. Alberto Severini, University of Alberta, Edmonton, Alberta. Human herpesvirus type 6 (HHV-6) infected T lymphoblastoid (HSB-2) cell extracts were obtained from Dr. Lung-Ji Chang, University of Alberta, Edmonton, Alberta.

Stably transfected HeLa S3 cells were grown to ~70% confluence in 25 cm^2 flasks and were either uninduced or induced to express F-ICP22 protein by rinsing the flasks with normal growth media (D-MEM, 10% newborn calf serum, 1% penicillin, 1% streptomycin, 200 µg/ml hygromycin, 1 mg/ml geneticin, and 1 µg/ml tetracycline) or media lacking tetracycline (D-MEM, 10% fetal calf serum, 1% penicillin, and 1% streptomycin) respectively. The control cell line 33 was uninduced or induced with its normal growth media (D-MEM, 10% newborn calf serum, 1% penicillin, 1% streptomycin, 1 mg/ml geneticin), or media lacking tetracycline (D-MEM, 10% fetal calf serum, 1% penicillin, and 1% streptomycin). The cells were incubated after rinsing the cells with media at 37°C for 24 hr. Whole cell extracts were prepared as described above and resuspended in 250 µl Laemmli buffer per 5 x 10^6 cells.

2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 200 V. SDS-PAGE was performed using the Mini-PROTEAN II dual slab cell apparatus from Bio-Rad Laboratories, Mississauga, Ontario. The separating gel used in all experiments, except for SDS-PAGE of p350/DNA-PKcs, consisted of 0.1% volume/volume (v/v) N,N,N,N',N'-tetramethylethylenediamine (TEMED), 0.045% fresh ammonium persulfate, 375 mM Tris-HCl pH 8.8, 0.1% SDS, and the appropriate amount of 30% acrylamide/0.8% bis-acrylamide solution for the required percentage of gel. The separating gel used for SDS-PAGE of p350/DNA-PKcs consisted of 0.05% v/v TEMED, 0.037% fresh ammonium persulfate, 40 mM Tris-HCl pH 8.8, 0.1% SDS, 8.4% acrylamide and 0.07% bis-acrylamide. Separating gels were layered with water saturated isobutanol and allowed to polymerize for 30 min. The stacking gel was then poured. Stacking gels contained 0.1% v/v TEMED, 0.045% ammonium persulfate, 125 mM Tris-HCl pH 6.8, 0.1% SDS, 3.8% acrylamide and 0.1% bis-acrylamide and were allowed to polymerize for 30 min.

Whole cell extracts were syringed 10x to decrease the viscosity of the sample and to shear the DNA prior to loading on SDS polyacrylamide gels. In some experiments, the extracts were treated with deoxyribonuclease (DNase) I, purchased from Worthington Biochemical Corporation, Freehold, NJ. An equal volume (40 µl) of whole cell extract was loaded in each lane. Other samples loaded onto SDS polyacrylamide gels included *in vitro* transcription nuclear extracts prepared from HeLa cells (60 µg/lane), purchased from Promega Corporation, Madison, WI, and kaleidoscope standard molecular weight markers (5 µl/lane) purchased from Bio-Rad Laboratories. The samples were boiled for 10 min at 100°C prior to loading onto SDS polyacrylamide (6%-12%) gels.

Gels were run in buffer containing 125 mM Tris-HCl, 0.96 M glycine, and 0.5% SDS (overall pH 8.3) at 100 V for 15 min and then at 200 V until the appropriate protein separation was reached.

3. Protein transfer

The proteins were transferred to Hybond (ECL) nitrocellulose membranes (0.45 µm pore size) from Amersham, Oakville, Ontario using the Mini transblot electrophoretic transfer cell from Bio-Rad Laboratories for 3 hr at 100 V or for 16 hr at 20 V. The transfer buffer used in all experiments, except for Western blotting of p350/DNA-PKcs, consisted of 25 mM Tris, 192 mM glycine, 20% methanol and 0.05% SDS (overall pH 8.3). The transfer buffer used in Western blotting of p350/DNA-PKcs, specific for improving transfer of larger MW proteins, consisted of 50 mM Tris, 40 mM glycine, 20% methanol, and 0.036% SDS.

4. Antibody incubation and protein detection

The nitrocellulose filters were blocked for > 2 hr with 10% weight/volume (w/v) powdered milk and 3% BSA in PBS-T. The blots were then washed with PBS-T containing 0.1% to 0.5% SDS 3x for 5 min. Variable amounts of SDS were used in the PBS-T rinses depending on the amount of nonspecific background signal observed for each specific primary antibody. The filters were probed with primary antibodies for 1 hr at room temperature and washed as described above. The blots were then incubated with horseradish peroxidase-conjugated goat anti-mouse or horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies for 1 hr at room temperature. The blots were washed with PBS-T containing SDS 6x for 5 min. Proteins were detected using the enhanced chemiluminescence (ECL) method from Amersham. Bands on immunoblots were visualized by autoradiography.

5. Stripping Western blots

The nitrocellulose filters were incubated in hybridization buffer consisting of 62.5 mM Tris-HCl pH 6.7, 2% SDS, and 100 mM β-mercaptoethanol at 50°C. Filters were incubated for 20 min to 2 hr depending on the intensity of the hybridization and background signals. The filter was rinsed six times in PBS-T (50 ml) at room temperature, followed by four washes at 50°C: 400 ml PBS-T for 20 min, 300 ml PBS-T for 15 min, and two washes of 300 ml PBS-T for 10 min at each. The rinsing steps were required to remove all traces of β-mercaptoethanol from the blot. The blot was blocked for > 2 hr with 10% milk and 3% BSA in PBS-T and then washed and probed with other primary antibodies as described above.

E. Southern blot analysis

1. DNA extraction

Stably transfected HeLa S3 F-ICP22 cell lines were grown to ~70% confluence in 75 cm^2 flasks and were harvested by treating the culture with trypsin to loosen the cells from the flask. An equal volume of growth media was added to the trypsinized cells and the cells were pelleted by centrifugation at 1000 rpm for 5 min at 4°C. The pellet was resuspended in 10 ml PBS and repelleted by centrifugation as described above. The pellet was resuspended in 400 µl PBS. The cells were lysed in > 5 volumes (2.5 ml) of 4 M guanidinium isothiocyanate solution (in 4.7 mg/ml N-lauroyl sarcosine, 4.7 mM sodium citrate pH 7.2, and 1.75% (v/v) β-mercaptoethanol).

Cesium chloride at a concentration of 1 g cesium chloride/2.5 ml lysate was added. For the 2.5 ml guanidinium isothiocyanate solution, 1 g of cesium chloride was added and mixed by hand. The sample was layered on top of a cesium chloride cushion (5.7 M cesium chloride and 0.1 M EDTA) and ultracentrifuged at room temperature overnight at 35,000 rpm in a swinging bucket rotor (SW55 Ti). The viscous DNA band approximately 1/2 to 1/3 down the gradient was removed. Water was added to a total volume of 6 ml. Chloroform/isoamylalcohol (24:1) (4 ml) and phenol (4.5 ml) was added and the sample was mixed thoroughly with a vortex. Separation between the organic and aqueous layer was performed by centrifuging the sample at 3,500 rpm for 20 min at room temperature. The sample was extracted two additional times as described above using chloroform/isoamylalcohol (24:1) and phenol and then extracted with chloroform/isoamylalcohol (24:1) alone. The sample was centrifuged at 3,500 rpm for 10 min at room temperature. The aqueous layer was removed and the sample was precipitated with ethanol. The DNA was pelleted by centrifuging at 6,000g for 25 min at 4°C using the Sorval centrifuge (SA600 rotor). In the final step, the DNA was resuspended in TE pH 7.5.

2. Southern blotting

Isolated HeLa S3 F-ICP22 DNA was digested with *Hinc* II. The *Hinc* II restriction sites are present in the F-ICP22 DNA sequence at 3461, 3512, 4114, and 4702 (Fig. 9.9). A positive control containing a linearized *EcoN* I 8Flag/ICP22 (Bam) (7.6 kb) DNA fragment was also run. The 8Flag/ICP22 (Bam) plasmid was prepared by Alison Kilvert, University of Alberta, Edmonton, Alberta and contains F-ICP22 inserted into pUC19 at the *Bam* HI site. Digested DNA purified by phenol extraction, chloroform/isoamylalcohol (24:1) extraction, and ethanol precipitation.

The digested DNA was run on a 17 cm long 0.8% agarose gel at 100 V for 3 hr until the 2 kb marker migrated 8 cm from the well. Twenty μg of HeLa S3 F-ICP22 DNA and 23.76 pg of positive control DNA was loaded on the gel. The 24 pg of positive control corresponds to one insertion of the F-ICP22 gene incorporated into the HeLa S3 cells. In addition, λ *Hind* III DNA markers were loaded on the gel.

The gel was prepared for blotting by incubating at room temperature for 30 min in 0.4 N sodium hydroxide and 0.6 N sodium chloride. The gel was next incubated as described above in 1.5 M sodium chloride and 0.5 M Tris-HCl pH 7.5. The gel was blotted with Gene Screen Plus nitrocellulose membrane by capillary action overnight at room temperature.

The filter was treated for 1 min in 0.4 N sodium hydroxide and then for 1 min in 0.2 M Tris pH 7.5 and 2x SSC. The filter was air dried and then UV crosslinked using the Stratalinker (Stratagene, Aurora, Ontario). The blot was baked at 80°C under vacuum for 2 hr and wet with 0.1x SSC, 0.1% SDS, and 0.2 M Tris-HCl pH 7.5. The solution was drained and the filter was prehybridized with modified Westneat solution (6.6% SDS, 250 mM sodium phosphate pH 7, 5x Denhardts, and 1 mM EDTA) for > 1 hr at 55°C in a hybridization oven. After heating at 95°C for 10 min on a heating block, the DNA probe was added to the blot at a concentration of 10^6 cpm/ml in 100 µg/ml salmon sperm DNA and modified Westneat solution.

The DNA probe was prepared from the 8Flag/ICP22 (Bam) by excising a 857 bp DNA *Bgl* II-*Xho* I fragment in the F-ICP22 DNA sequence between 1262 and 2122 (Fig. 9.9). The DNA fragment was isolated from a 0.7% low melt agarose gel (Nu sieve GTG agarose) and purified using the Wizard DNA purification system purchased from Promega. The DNA band was excised from the low melt gel and centrifuged at 13,000 rpm for 1 min at room temperature. The maximum volume of the gel was 300 µl. The gel was heated for 2 min at 70°C to dissolve the agarose and 1 ml of DNA purification resin was added and vortexed. The resin solution was passed over a Wizard mini-column under vacuum. The mini-column was washed with 2 ml 80% isopropanol and dried. The DNA was eluted from the mini-column by adding 50 µl of TE pH 7.5 at 65°C and then centrifuging the mini-column at 13,000 rpm for 1 min at room temperature. The DNA was purified further by phenol extraction, chloroform/isoamylalcohol (24:1) extraction, and ethanol precipitation. The DNA probe was radiolabeled for Southern blotting by random priming methods.

The DNA probe was hybridized to the filter for 4 d at 55°C in a hybridization oven. The filter was washed 2x at room temperature for 5 min each in 2x SSC containing 0.2% SDS. The blot was washed twice at 55°C for 20 min in 0.2x SSC containing 0.2x SDS in a shaking water bath. Bands were visualized by autoradiography.

F. Northern blot analysis

1. RNA extraction

Stably transfected HeLa S3 F-ICP22 cells were grown to ~70% confluence in 75 cm^2 flasks and were uninduced and induced for F-ICP22 RNA expression as described in section D.1. Cells were harvested and lysed in guanidinium and RNA was purified over a cesium chloride gradients as described in section E.1. After the ultracentrifugation step, the supernatant was removed carefully, and the RNA pellet was resuspended in TE pH 7.5. The RNA was purified by phenol extraction, isoamylalcohol/chloroform (24:1) extraction and ethanol precipitation. In the final step, RNA was resuspended in TE pH 7.5.

2. Northern blotting

Twenty µg of HeLa S3 F-ICP22 RNA was ethanol precipitated. A positive control contained a linearized *EcoN* I 8Flag/ICP22 (Bam) (7.6 kb) DNA fragment as described in section E.2. Lambda *Hind* III DNA markers and a 0.16 kb - 1.77 kb RNA ladder from Gibco BRL were run as markers. All RNA and DNA samples were resuspended in 1x 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (2 mM MOPS pH 7.2, 5 mM sodium acetate, and 1 mM EDTA), 17.6% formaldehyde (37% w/w stock), and 50% fresh formamide. The samples were heated at 65°C for 10 min prior to loading on the gel.

The nucleic acids were run on a 0.7% agarose/formaldehyde gel (1x MOPS buffer and 18% of formaldehyde [37% w/w stock]) in formaldehyde running buffer (1x MOPS buffer and 18% of formaldehyde [37% w/w]) at 150 V for ~3 hr until the bromophenol blue marker migrated 7.5 cm from the well. To visualize the MW markers, the gel strip containing the markers was removed and stained with ethidium bromide solution (0.5 µg/ml ethidium bromide and 0.1 M ammonium acetate) overnight. The markers were destained in 0.1 M ammonium acetate for 2 hr and visualized in UV light.

The gel was rinsed 2x with water and then treated with 50 mM sodium hydroxide and 10 mM sodium chloride for 30 min. The gel was treated with 100 mM Tris-HCl pH 7.5 for 30 min and then 10x SSC for 30 min. The gel was blotted with Gene Screen Plus nitrocellulose membrane by capillary action > 16 hr at room temperature.

The filter was rinsed with 2x SSC and then air dried. The blot was baked at 80°C under vacuum for 2 hr and UV crosslinked using the Stratalinker (Stratagene). The filter was wet for 1 min in 2x SSC and then prehybridized with modified Westneat solution for > 1 hr at 55°C in a hybridization oven. After heating at 95°C for 10 min, the DNA probe was added to the blot at a concentration of 2×10^6 cpm/ml in 100 µg/ml salmon sperm DNA and modified Westneat solution. The probe used for Northern blotting was described in section E.2.

The DNA probe was hybridized to the filter for 3 d at 55°C in a hybridization oven. The filter was washed 2x at room temperature for 5 min in 2x SSC containing 0.2% SDS. The blot was washed twice at 55°C for 20 min in 0.2x SSC containing 0.2x SDS in the shaking water bath. Bands were visualized by autoradiography.

G. Calf intestinal alkaline phosphatase (CIAP) reactions

1. RNAP II large subunit

Mock infected and WT HSV-1 infected whole cell extracts in Laemmli sample buffer were dialyzed against CIAP buffer consisting of 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) pH 7.9, 20 mM magnesium chloride, 40 mM potassium chloride, and 0.2 mM PMSF. Dialysis was performed with 50 µl of sample on spin dialysis filters (VM: 0.05 µM pore size) from Millipore Corporation for 2 hr at 4°C. Dialyzed samples were untreated or treated with 50 units of CIAP (in 30 mM triethanolamine pH 7.6, 3 M sodium chloride, 1 mM magnesium chloride, and 0.1 mM zinc chloride) from Gibco BRL for 35 min at 37°C, after which an equal amount of 2x cracking buffer (120 mM Tris-HCl pH 6.8, 2% SDS, 20% glycerol, and 0.02% bromophenol blue) was added. The untreated and CIAP-treated samples were then analyzed by Western blotting to detect the RNAP II large subunit.

2. FLAG ICP22 (F-ICP22)

Laemmli extracts prepared from F22 infected HeLa S3 cells (30 µl) were treated with 30 units of CIAP for 35 min at 37°C. The CIAP treated samples were analyzed by Western blotting to detect FLAG ICP22 (F-ICP22).

H. Protein synthesis inhibitor studies

Vero cells were pretreated in D-MEM containing 10% heat-inactivated newborn calf serum, 1% penicillin, 1% streptomycin, and 50 µg/ml of the protein synthesis inhibitor cycloheximide (Sigma Chemical Company) for 1 hr. The cells were then mock infected or infected with WT HSV-1 in the continuous presence of 50 µg/ml cycloheximide for 6 hr. Cycloheximide treatment was reversed by washing the cells three times in medium without the drug and then incubating the cells in medium without the drug for 2 hr at 37°C. Whole cell extracts were prepared and analyzed by Western blot analysis as described above. Another protein synthesis inhibitor employed in these studies was anisomycin, which was used at a concentration of 100 mM and purchased from Sigma Chemical Company.

I. Phosphoamino acid analysis

1. Preparation of ^{32}P-labeled nuclear lysates

The procedure for the preparation of ^{32}P-labeled nuclear lysates was a modified method described previously (Cadena and Dahmus, 1987; Baskaran et al., 1993). Vero cells were grown to ~70% confluence in 150 cm^2 flasks (2 x 10^7 to 8 x 10^7 cells). Cells were either mock infected or infected with WT HSV-1 (KOS1.1). The virus inoculum was allowed to adsorb to the cells for 1 hr at 37°C. The inoculum was removed and the cells were rinsed with TBS (10 ml) 3x. Phosphate depleted media (D-MEM without sodium phosphate) supplemented with 10% dialyzed fetal bovine serum, 10% penicillin, 10% streptomycin, and 50 µCi/ml of ^{32}P-orthophosphate (Amersham) was added to the cells. The cells were then incubated at 37°C for an additional 5 hr. Two hr prior to harvesting the cells, a final concentration of 1 mM sodium orthovanadate was added to the cell culture.

All buffers used in harvesting the cells contained the following protease and phosphatase inhibitors: 1 mM sodium orthovanadate, 25 mM sodium fluoride, 50 mM sodium phosphate, 50 mM sodium pyrophosphate, 0.2 mM pefabloc (Boehringer Mannheim, Laval, Quebec), 10 µM benzamidine, 10 µg/ml 1, 10-phenanthroline, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, and 1 mM PMSF. The inhibitors were added to the buffers immediately prior to use. Harvesting the cells was carried out at 4°C. Cells were scraped in PBS and collected by centrifugation at 800g for 5 min and were resuspended in 10 mM Tris-HCl pH 7.9, 1 mM calcium chloride, 1.5 mM magnesium chloride, 0.25 M sucrose, and 0.5% Triton X-100. Cells were lysed (15 strokes) with a hand homogenizer from Bellco Glass Inc., Vineland, NJ.

The nuclei were collected by centrifugation at 800g for 5 min at 4°C. The nuclei were rinsed 2x with 10 mM Tris-HCl pH 7.9, 1 mM calcium chloride, 1.5 mM magnesium chloride, and 0.25 M sucrose. The nuclei were pelleted at 800g for 5 min at 4°C and resuspended in 25 mM Tris-HCl pH 8, 2.5 mM magnesium acetate, 2 mM calcium chloride, 0.05 mM EDTA, 0.1 mM dithiothreitol (DTT), and 12.5% glycerol at a final concentration of 10^8 nuclei/ml. The nuclei were incubated in 100 µg/ml ribonuclease A (RNase A) (prepared in 10 mM Tris-HCl pH 7.5 and 1.5 mM sodium chloride), from Sigma Chemical Company and 5 µg/µl DNase I (prepared in 20 mM sodium acetate, 5 mM calcium chloride, and 50% glycerol) for 30 min at 4°C. An equal volume of 2% SDS was added and the sample was boiled at 100°C for 3 min. The sample was cooled at room temperature and centrifuged at 15,600g for 3 min at 4°C. The supernatant was collected and dispensed into fractions containing approximately 5 x 10^6 nuclei/tube. The nuclear lysate was diluted into a RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, and

0.1% SDS). In addition, protease and phosphatase inhibitors as described earlier in this section were added to the nuclear lysate.

2. Immunoprecipitation of the large subunit of RNAP II

One ml of ^{32}P-labeled nuclear lysate (5 x 10^6 nuclei) was incubated with either 8WG16 (17.28 µg) or ARNA3 (5 µg), antibodies which recognize the large subunit of RNAP II for 3 hr at 4°C. The sample was mixed during the incubation period on a nutator.

During immunoprecipitations with ARNA3, a bridger antibody (5 µg) was added after the 3 hr incubation period. The bridger antibody improved the efficiency of the immunoprecipitation process and was a rabbit anti-mouse immunoglobulin G (IgG) (heavy and light chains) purchased from Jackson ImmunoResearch Laboratories Inc. Immunoprecipitations with 8WG16 did not require the use of the bridger antibody.

Pansorbin cells (Staphylococcus aureus protein A, Cowan I strain) from Calbiochem, La Jolla, CA, were used to immunoprecipitate antigen-antibody complexes. Pansorbin cells (50 µl) that were rinsed in RIPA buffer (containing the following protease and phosphatase inhibitors: 1 mM sodium orthovanadate, 25 mM sodium fluoride, 50 mM sodium phosphate, 50 mM sodium pyrophosphate, 0.2 mM pefabloc, 10 µM benzamidine, 10 µg/ml 1, 10-phenanthroline, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, and 1 mM PMSF) were added to the antibody-antigen reaction mixture and incubated at 4°C for 30 min with mixing. The immunoprecipitate was collected by centrifugation for 1 min at 10,000 rpm at 4°C. The supernatant was decanted and the pellet was washed 3x with RIPA buffer (containing protease and phosphatase inhibitors as described above). The immunoprecipitates were collected by centrifugation for 1 min at 10,000 rpm at 4°C. The pellet was resuspended in 40 µl of 2x cracking buffer (containing fresh 1% β-mercaptoethanol) and heated at 85°C for 10 min. The pellet was precipitated by centrifuging for 1 min at 10,000 rpm at 4°C, and the supernatant containing the RNAP II large subunit of RNAP II was collected.

As a control, Pansorbin cells were incubated with the ^{32}P-labeled nuclear lysate in the absence of 8WG16 or ARNA3 to ensure that the large subunit of RNAP II was specifically immunoprecipitated by the antibodies and not by the Pansorbin cells. Other controls included incubating the bridger antibody with the ^{32}P-labeled nuclear lysate in the absence of 8WG16 or ARNA3 to ensure that the large subunit of RNAP II was not precipitated by the bridger antibody.

3. HCl digestion and phosphoamino acid identification

The RNAP II immunoprecipitates isolated from ^{32}P-labeled nuclear lysates were separated by SDS-PAGE (6%) as described in this chapter (section D.2). The proteins were transferred to Immobilon-P (PVDF) membrane (Millipore Corporation, Beford, MA) for 3 hr at 100 V as described earlier in this chapter (section D.3).

The blot was rinsed with distilled water, covered with Saran wrap while still moist, and exposed to film overnight. The ^{32}P-labeled immunoprecipitated RNAP II large subunit was identified on the blot. The small band of Immobilon-P containing RNAP II large subunit was excised from the rest of the blot and digested with 600 µl of 6 N HCl for variable periods of time (1 hr - 5 hr) at 110°C. Shorter hydrolysis times (1 hr) in 6 N HCl at 110°C increases the yield of phosphotyrosine. Longer hydrolysis times, 3 and 5 hr in 6 N HCl at 110°C, increases the yields of phosphoserine and phosphothreonine respectively (Cooper et al., 1983; Duclos et al., 1991). The volatile HCl was evaporated using a speed vacuum at room temperature.

The amino acids were resuspended in water and mixed with 4 µl of a 10 mM stock of unlabeled phosphoserine, phosphothreonine, and phosphotyrosine. These unlabeled phosphoamino acids were used as markers to identify ^{32}P-labeled amino acids. The sample was spotted onto 10 cm by 10 cm thin layer plastic sheets (cellulose, 0.1 mm layer, without fluorescence indicator) purchased from EM Separations (Merck), Gibbstown NJ. Samples were applied to plates at the origin, 1.5 cm horizontal (X-axis) and 1.5 cm vertical (Y-axis) from the edge of the plate, 0.5 µl at a time. Between sample applications, the origin was dried. It was important to ensure that the sample was spotted onto one small single point to prevent bleeding of the sample and poor resolution of the amino acids. In addition, 0.5 µl of 1 µg/µl (0.5 µg) xylene cyanole dye was spotted 8 cm from the origin on the X-axis to serve as a general marker for sample migration.

The amino acids were separated by two-dimensional thin layer electrophoresis at 4°C. The first dimension was run in pH 1.9 buffer (2% formic acid and 7.8% acetic acid) along the Y-axis at 490 V, until the xylene cyanole dye migrated 8 cm. The sheet was air dried at room temperature. The thin layer sheet was spotted with 0.5 µl of 1 µg/µl (0.5 µg) xylene cyanole dye at the origin and used as a marker for sample migration. The second dimension was run in pH 3.5 buffer (0.5% pyridine and 5% acetic acid), along the X-axis at 300 V until the xylene cyanole dye migrated 3.5 cm from the origin. The sheet was air dried at room temperature.

The thin layer sheet was sprayed with a fine mist of ninhydrin (0.2% weight/volume ethanol) in the fumehood. The sheet was dried and the phosphoserine, phosphothreonine, and phosphotyrosine controls were visualized as purple/pink spots. The thin layer sheet was analyzed by autoradiography and ^{32}P-labeled amino acids were identified. In addition, ^{32}P-labeled amino acids were quantitated by PhosphorImager and Fujix BAS100 bio-imaging analyzer with MacBAS imaging software.

J. Nuclear run-on transcription assay

1. Nuclei isolation

Nuclei were isolated for nuclear run-on transcription assays as previously described (Spencer et al., 1990). Vero, HeLa S3 or HEL 299 cells were grown to ~70% confluence in 150 cm^2 flasks. The cells were mock infected or infected with either WT HSV-1 (KOS1.1), 22/*n*199, *d*UL13 or F22. At 3, 6, and 9 hr post-infection, cells were harvested by treating the culture with trypsin to loosen the cells from the flask. An equal volume of D-MEM containing 10% newborn calf serum, 1% penicillin and 1% streptomycin was added to the trypsinized cells and the cells were pelleted by centrifugation at 1,000g for 5 min at 4°C. The pellet was washed 2x in 10 ml of PBS containing 0.5 mM PMSF. The cells were counted with a haemocytometer and then pelleted by centrifugation as described above. Cells were resuspended in 10 ml reticulocyte saline buffer (RSB) (10 mM sodium chloride, 10 mM Tris-HCl pH 7.4, and 5 mM magnesium chloride). The cells were pelleted by centrifugation at 1,000g for 5 min at 4°C, and resuspended in 2 ml of RSB. Twenty ml of RSB containing 0.5% Nonidet P40 was added, and the cells were lysed using a hand homogenizer (10 strokes). The nuclei were collected by centrifugation at 2,100 rpm for 5 min at 4°C. The supernatant was removed, and the pellet was resuspended in 210 µl of nuclear freezing buffer (50 mM Tris-HCl pH 8.0, 5 mM magnesium chloride, 40% glycerol and 0.5 mM fresh DTT) per 1-2 x 10^7 cells. The resuspended nuclei were stored at -70°C.

2. Nuclear run-on transcriptions

Nuclear run-on transcription assays were performed on equal numbers of nuclei per sample, as previously described (Spencer et al., 1990). Transcription was allowed to proceed by incubating isolated nuclei in a final concentration of 35 mM Tris-HCl pH 8.0, 3.5 mM magnesium chloride, 148 mM potassium chloride, 0.25 mM ATP, 0.25 mM GTP, 0.25 mM CTP and 300 µCi ^{32}P-α-UTP (Amersham) for 30 min at 30°C. The reaction was terminated by incubating with 125 µg of DNase I for 10 min at 30°C. An additional 75 µg of DNase I was added to the sample, and the reaction was incubated for an additional 10 min at 30°C. The sample was diluted to 1% SDS, 10 mM Tris-HCl pH 7.5, and 5 mM EDTA and treated with 100 µg proteinase K for 45 min at 45 °C. The RNA was purified by phenol extraction, chloroform/ isoamylalcohol (24:1) extraction and isopropanol/ammonium acetate precipitation. The RNA was resuspended in 100 µl TE (10 mM Tris-HCl pH 7.5 and 1 mM EDTA) and separated from unincorporated ^{32}P-α-UTP by passing the sample over a 1 ml G50 Sephadex spin column at 1,200 rpm for 4 min. The eluted RNA was diluted in hybridization buffer (10 mM Tris-HCl pH 7.4, 1% SDS, 10 mM EDTA, 250 µg/ml *E. Coli* RNA, 0.3 M sodium chloride, 1x Denhardts and 0.25% (w/v) powdered milk).

RNA was hybridized to Gene Screen Plus membranes containing single-stranded M13 bacteriophage DNA probes specific for various viral and cellular genes. DNA probes (1 μg per slot) were first diluted in 1x SSC (150 mM sodium chloride and 15 mM sodium citrate dihydrate) and slot blotted to the filters using the Bio-Dot SF apparatus (Bio-Rad Laboratories) under low vacuum. Filters were dried slightly and UV cross-linked using a Stratalinker (Stratagene). Filters were then dried at room temperature and baked at 80°C under vacuum for 2 hr.

The nitrocellulose filters were prehybridized with hybridization buffer for 2 hr at 65°C. These filters were then hybridized to ^{32}P-labeled RNA, synthesized from the nuclear run-on transcription reactions, for 48 hr at 65°C. The filters were rinsed twice at room temperature for 5 min in 1x SSC containing 0.1% SDS and then incubated at room temperature for 30 min in 2x SSC containing 2 μg/ml RNase A for 30 min at room temperature; the RNase A washes removed non-hybridizing portions of ^{32}P-labeled RNAs. The filters were washed twice at room temperature for 5 min in 1x SSC containing 0.1% SDS and then twice at 65°C for 30 min in 1x SSC containing 0.1% SDS. Filters were analyzed by autoradiography. In addition, radioactivity hybridizing to each probe was quantitated with a Fujix BAS100 bio-imaging analyzer with MacBAS imaging software.

3. Viral and cellular DNA probes

The DNA probes were designed to detect sense or antisense transcription in the gene of interest. The probes were constructed by cloning DNA fragments into either M13mp18 or M13mp19. Probes that detect RNA from HSV-1 genes encoding the ICP4, ICP8, ICP5, and gC genes have been described previously (Godowski and Knipe, 1986) and were obtained from Dr. Stephen Rice, University of Alberta, Edmonton, Alberta. The probes for ICP27 contain a 1.2 kb *Bam* HI-*Sal* I insert from the 5′ half of the ICP27 gene and were obtained from Dr. L. Su and Dr. David Knipe, Harvard Medical School, Boston, Massachusetts. The probes for DNA polymerase were from Dr. Donald Coen and Dr. Steve Weinheimer, Harvard Medical School and contained a 0.6 kb *Bgl* II-*EcoR* I fragment of the 5′ half of the DNA polymerase gene. The probes for the VP16 gene contained a 1.2 kb *Sal* I insert taken from the VP16 (UL48) gene. The probes for the UL36 gene contained a 1.3 kb *Hind* III-*Bam* HI insert from the 5′ end of the UL36 gene. Both the VP16 and UL36 probes were obtained from Dr. Stephen Rice.

The c-myc exon 1 probe contained a 445 base *Xho* I - *Pvu* II fragment (+66 to +511) from the human c-myc gene (Spencer et al., 1990). The c-myc intron 1 probe contained a 606 bp *Sst* I fragment (+936 to +1542) of the human c-myc gene (Spencer et al., 1990). The fos 5′ probe was a 842 bp *Nar* I fragment (-82 to +760) consisting of exon 1 and a portion of intron 1 of the human c-fos genomic DNA. The fos 3′ probe was a 491 bp *Apa* I fragment (+1905 to +2396) containing exon 4 of the human c-fos genomic DNA. The probe for γ-actin 5′ was a 583 bp *Bam* HI - *Bgl* I fragment (-100 to +483) containing exons 1 to 4 of a human γ-actin cDNA. The probe for γ-actin 3′ was a 600 bp *Bgl* I - *Sca* I fragment (+483 to +1083) consisting of exons 4 to 6 of a human γ-actin cDNA (Erba et al., 1988). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a 979 bp fragment (+44 to +1023) of a human GAPDH cDNA (Arcari et al., 1984). The probe for histone H2b was a 302 bp *BstE* II fragment (+110 to +412) of the chicken histone H2b gene (Spencer and Kilvert, 1993). All probes that detected RNA transcripts arising from cellular genes were provided by Alison Kilvert and Dr. Charlotte Spencer, University of Alberta, Edmonton, Alberta.

K. Immunoprecipitations of ICP22 and ICP4

1. Nuclear extract preparation

Nuclear extracts were prepared for immunoprecipitations by the method of Shapiro et al. (1988). HeLa S3 cells in logarithmic phase were grown as a suspension culture in spinner flasks to a concentration of 7 x 10^6 cells/ml at 37°C. Cells (1 L) were uninfected or infected with either W T HSV-1 (KOS1.1), *d*120, or F22. The virus inoculum was added directed to the HeLa S3 cells at a MOI of 5 or 10 and cells were incubated at 37°C for 12 hr. An MOI of 5 was used in some experiments. The cells were efficiently infected at an MOI of 5.

The cells were harvested at 12 hr post-infection. All buffers contained the following protease inhibitors: PMSF (200 µg/ml, 86 mM), benzamidine (20 μg/ml, 12.8 mM), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (2 μg/ml) and leupeptin (2 μg/ml). In all solutions containing DTT, the DTT was added immediately prior to use of the buffer. Cells were pelleted by low-speed centrifugation at 170g for 10 min at 4°C. The cells were washed twice in 25 and 10 volumes of PBS and then repelleted as described above. The pellet was resuspended in a 5 packed cell volume (PCV) of hypotonic buffer (10 mM HEPES pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 10 mM potassium chloride). The cells were pelleted by low-speed centrifugation at 300g for 10 min at 4°C. The pellet was resuspended in 2 PCVs of hypotonic buffer and lysed by hand with a tissue grinder (40 strokes) after which 0.1 volumes of sucrose restore buffer (9 volumes of 75% RNase-free sucrose and 1 volume of 10x salts solution [500 mM HEPES pH 7.9, 7.5 mM spermidine, 1.5 mM spermine, 100 mM potassium chloride, 2 mM EDTA, and 10 mM DTT]) was added in order to prevent nuclear lysis.

The nuclei were pelleted in a Sorval centrifuge at 16,000g for 30 sec at 4°C. The pellet was resuspended at a concentration of 3 ml/10^9 cells in nuclear resuspension final buffer (9 volumes of nuclear resuspension buffer [20 mM HEPES pH 7.9, 0.75 spermidine, 0.15 mM spermine, 0.2 EDTA, 2 mM EGTA, 2 mM DTT, and 25% glycerol] and 1 volume of saturated ammonium sulfate) and incubated for 30 min at 4°C. Chromatin and cell debris were precipitated by ultracentrifugation in a Ti90 rotor at 60,000 rpm for 25 min at 4°C. The supernatant was removed and subjected to further ammonium sulfate precipitation. Liquid ammonium sulfate was slowly added in a dropwise fashion over a period of 20 min to a concentration of 0.33 g/ml. The solution was slowly mixed on a shaker for 30 min at 4°C. The precipitate was pelleted by ultracentrifugation in a Ti90 rotor at 45,000 rpm for 10 min at 4°C. The pellet was resuspended at a concentration of 1 ml/10^9 cells in nuclear dialysis buffer (20 mM HEPES pH 7.9, 20% glycerol, 100 mM potassium chloride, 0.2 mM EDTA, 0.2 mM EGTA, and 2 mM DTT). The resuspended nuclear extract was dialyzed (3,500 kDa cut-off) for 5 hr against > 200 volumes of nuclear dialysis buffer. The dialysis tubing was purchased from Spectrum Medical Industries, Inc., Los Angeles, CA. The dialyzed nuclear extract was centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant was collected, dispensed into 10 μl fractions, and stored in liquid nitrogen. The protein concentration of the nuclear extract was determined by the Bradford assay (Bradford, 1976).

2. Unlabeled cellular lysate preparation

Cellular lysates were prepared for immunoprecipitations. Vero cells were grown to confluence in 25 cm^2 flasks and mock infected or infected with WT HSV-1, *d*120, or F22 for 5 to 6 hr. The cells were rinsed twice with PBS containing 0.1 µg/µl PMSF and 0.5 µg/µl TLCK. M2 lysis buffer (1% Triton X-100, 25 mM Tris-HCl pH 7.4, 300 mM sodium chloride, 1 mM calcium chloride, 10 µg/ml leupeptin, and 1 mM PMSF) (0.5 ml) was added to the cells, and the flasks were placed on ice for 30 min. During this 30 min period, the flasks were hand rocked several times for 15 sec. The sample was scraped and pelleted by centrifugation at 1,000 rpm for 10 min at 4°C. The supernatant was collected and stored at -70°C.

3. ^{35}S -Labeled cellular lysate preparation

^{35}S-Labeled cellular lysates were prepared for immunoprecipitations using the M2 antibody. Vero cells were grown to confluence in 25 cm^2 flasks and mock infected or infected with WT HSV-1, *d*120, or F22. At 2 hr post-infection, the cells were rinsed twice with labeling media (methionine minus media). To each flask, 1.5 ml of labeling media containing 20 µCi/ml ^{35}S methionine (^{35}S-methionine/cysteine) (NEN Research Products, Mississauga, Ontario) was added. The cells were harvested between 5 and 6 hr post-infection as described in section K.2.

4. Immunoprecipitation

Immunoprecipitation of F-ICP22 or ICP4 was performed using a modified method described previously (Shelness et al., 1994). The nuclear extract (30 µl or 60 µg - 450 µg protein diluted in 800 µl M2 lysis buffer (1% Triton X-100, 25 mM Tris-HCl pH 7.4, 300 mM sodium chloride, 1 mM calcium chloride, 10 µg/ml leupeptin, and 1 mM PMSF) and the cellular lysate (unlabeled or ^{35}S-labeled, 200 µl) prepared for immunoprecipitation were precleared by incubating the sample with 50 µl Omnisorb or Pansorbin cells (depending on the primary antibody used). The Omnisorb and Pansorbin cells were rinsed with M2 lysis buffer and blocked with 4% BSA prior to use. Preclearance was conducted for 30 min at 4°C with mixing. The Omnisorb or Pansorbin cells were removed from the cell lysate by centrifugation at 10,000 rpm for 1 min at 4°C. The supernatant was collected and ready for immunoprecipitation.

Precleared supernatants were immunoprecipitated with M2, H1101, or H1114. The M2 antibody is an IgG_1 antibody that has a strong affinity for Omnisorb cells (protein G) (Calbiochem). H1101 and H1114, IgG and IgG_{2a} antibodies respectively, have high affinity for Pansorbin cells (protein A). For each immunoprecipitation, 3 µg of M2 antibody was used. Varying amounts of H1101 and H1114 were used. A series of control immunoprecipitations was also performed to ensure F-ICP22 and ICP4 immunoprecipitations were specific. Precipitations were performed with the cellular lysates and Omnisorb/Pansorbin cells in the absence of antibody to ensure that F-ICP22 and ICP4 were not precipitated nonspecifically by the Omnisorb/Pansorbin cells. In addition, the antibody was incubated with Omnisorb/Pansorbin cells in the absence of nuclear extract or cellular lysate to ensure that the antibody was not nonspecifically precipitating a protein from Omnisorb/Pansorbin cells. Immunoprecipitations were incubated overnight at 4°C with mixing.

Additional Omnisorb or Pansorbin cells (50 µl) that had been rinsed with M2 lysis buffer and blocked with 4% BSA were added to the reaction and incubated for 30 min at 4°C with mixing. The immunoprecipitates were pelleted by centrifugation at 10,000 rpm for 1 min at 4°C. The pellet was washed 4x with M2 lysis buffer and repelleted by centrifugation as described above. The pellet was resuspended in 35 µl M2 SDS sample buffer (50 mM Tris-HCl pH 8.0, 12% glycerol, 4% SDS, 0.1 M DTT, 1 mM EDTA, and 0.05% bromophenol blue). The samples were boiled for 10 min at 100°C and centrifuged as described above. The supernatant was collected and the protein extracts were analyzed by Western blotting as described in section D. The ^{35}S-labeled cellular lysates were not analyzed by Western blotting but by SDS-PAGE (12% acrylamide) as described in section D.2. The gel was dried and visualized by autoradiography.

CHAPTER THREE. MODIFICATION AND LOCALIZATION OF THE RNAP II LARGE SUBUNIT FOLLOWING HSV-1 INFECTION [1]

A. Introduction

During lytic infection, HSV-1 utilizes the host's RNAP II transcription machinery to express its own genome while repressing the expression of most cellular genes (Smiley et al., 1991; Spencer et al., 1997). The mechanism by which this occurs is unresolved. The IE genes are transcriptionally activated by VP16 and associated cellular factors at defined *cis*-acting elements present in each of the IE gene promoters. The mechanisms by which DE and L gene transcription is activated remain to be determined. Although certain *cis*-acting promoter elements, such as the Sp1 site and the TATA box (Coen et al., 1986; Homa et al., 1986; Johnson and Everett, 1986; McKnight and Tjian, 1986), lie upstream of DE and L genes, specific sequences that bind IE proteins and activate DE and L gene transcription are not present.

It has been proposed that the differential regulation of viral and cellular gene transcription may not be dictated by differences in their promoter sequences. Viral genes or promoters placed outside of their natural context in the cellular genome are regulated differently from viral genes in the viral genome (Silver and Roizman, 1985). Furthermore, cellular genes with their own promoters, when inserted into the HSV-1 genome, are not transcriptionally repressed like their cellular counterparts, but are instead activated like viral genes (Smiley et al., 1987; Panning and Smiley, 1989; Smibert and Smiley, 1990). These experiments suggest that activation of viral gene transcription and repression of cellular gene transcription may involve sequence-independent mechanisms such as the modification of RNAP II transcription apparatus, differences in higher

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order chromatin packaging, or transport of DNA and cellular transcription factors into specialized nuclear compartments (Smiley et al., 1991).

RNAP II is the central component of the mRNA transcription apparatus and is responsible for transcription of eukaryotic protein-coding genes. The enzyme consists of at least 10 subunits, ranging from 10 to 240 kDa (Sawadogo and Sentenec, 1990; Woychick and Young, 1990; Young, 1991; Dahmus, 1994; Kang and Dahmus, 1995; Dahmus, 1996). The largest subunit plays a direct role in transcription initiation and elongation and contains a remarkable feature not shared with the large subunits of RNAP I or III or with bacterial RNAPs (reviewed in Sawadogo and Sentenec, 1990; Zawel and Reinberg, 1995). This feature is the carboxy-terminal domain (CTD), which consists of up to 52 heptapeptide repeats of the consensus sequence YSPTSPS. The CTD is highly conserved in eukaryotes, is essential for cell viability and is the site of multiple phosphorylations on serine, threonine and tyrosine residues (Dahmus, 1994; Kang and Dahmus, 1995; Dahmus, 1996).

RNAP II exists in two forms *in vivo*, IIO and IIA, which differ in the extent of phosphorylation of the CTD. The large subunit of RNAP IIA (IIa) is nonphosphorylated and is thought to play a role in transcription initiation. The large subunit of RNAP IIO (IIo) is phosphorylated at up to 50 sites and is thought to be involved in transcription elongation. The transition between RNAP IIA and RNAP IIO occurs early in the transcription cycle and is coincident with the transition between initiation and elongation. After completion of a round of transcription, RNAP IIO is converted back to RNAP IIA before reinitiation of transcription can occur (Dahmus, 1994; Kang and Dahmus, 1995; Dahmus, 1996).

HSV-1 may modify RNAP II and the basal transcription machinery in order to activate viral transcription and suppress cellular transcription (Smiley et al., 1991). In this chapter, I report that HSV-1 infection modifies the large subunit of RNAP II and relocates the enzyme to sites of viral DNA replication.

B. RNAP II is localized to DNA replication compartments following HSV-1 infection

To determine the nuclear distribution of the RNAP II large subunit during HSV-1 infection, we performed indirect immunofluorescence experiments. Vero cells were mock infected or infected with HSV-1 (KOS1.1) for 5 hr. The cells were subsequently fixed and stained with either 8WG16 or ARNA3, antibodies which specifically recognize RNAP II large subunit. ARNA3 recognizes an epitope on the body of the large subunit of RNAP II and recognizes all forms of the RNAP II large subunit (Krämer et al., 1980; Krämer and Bautz, 1981). The 8WG16 antibody recognizes incompletely or unphosphorylated forms of the CTD of the RNAP II large subunit (Thompson et al., 1989; Thompson et al., 1990).

Immunofluorescence detection of RNAP II in mock infected cells revealed a diffuse nuclear distribution (Fig. 3.1B and 3.1F), with pronounced exclusion from the nucleoli (Fig. 3.1A, 1B, 1E, and 1F). Staining with 8WG16 showed a number of distinct bright speckles dispersed throughout the nucleus in mock infected cells (Fig. 3.1B). These speckles may represent localized concentrations of nonphosphorylated RNAP II, perhaps unengaged or nonelongating. The staining pattern observed with ARNA3 (Fig. 3.1F) was more uniform than that observed with 8WG16 (Fig. 3.1B). This may be due to the fact that ARNA3 recognizes all forms of the RNAP II large subunit while 8WG16 recognizes only incompletely phosphorylated forms.

After infection with HSV-1, the nuclear distribution of RNAP II was dramatically altered (Fig. 3.1D and 3.1H). After 5 hr of infection, the majority of RNAP II was localized into a number of distinct subnuclear foci. As infection proceeded, these foci fused into large globular structures which occupied most of the nucleus (data not shown). With the 8WG16 antibody, the overall level of staining for RNAP II large subunit after virus infection appeared to be greater than before infection (Fig. 3.1D versus 3.1B). No similar increase in staining intensity was observed after virus infection with the ARNA3 antibody (Fig. 3.1F versus 3.1H), suggesting that RNAP II large subunit was recognized more efficiently by 8WG16 after infection, possibly due to an alteration in CTD phosphorylation.

Previous studies examining nuclear structure during HSV-1 infection have shown that large globular compartments characteristically form within 6 hr of infection. These compartments increase in size throughout infection (Quinlan et al., 1984), are sites of DNA replication, and have been termed DNA replication compartments (Quinlan et al., 1984; Randall and Dinwoodie, 1986; de Bruyn Kops and Knipe, 1988). The localization of cellular and viral regulatory proteins to replication compartments has been previously described (Randall and Dinwoodie, 1986; Knipe et al., 1987; Wilcock and Lane, 1991).

We then asked whether the sites of RNAP II localization in infected cells were identical to viral DNA replication compartments. We performed double immunofluorescence experiments to determine whether the RNAP II large subunit colocalized with ICP8, the major HSV-1 DNA binding protein which associates with replication compartments. ICP8 is a DE gene product that is essential for HSV-1 DNA replication and for the formation of viral replication compartments (Quinlan et al., 1984). ICP8 exhibits DNA binding activity without any apparent sequence specificity and has preferential affinity for single-stranded DNA (Knipe and Spang, 1982; Conley et al., 1981). Vero cells were infected with HSV-1 for 5 hr, fixed, and then stained with primary antibodies, 3-83, an antiserum directed against ICP8, and either 8WG16 or ARNA3. Double immunofluorescence staining patterns for both 8WG16 and ARNA3 showed that RNAP II and ICP8 distributions after infection were coincident (Fig. 3.2), indicating that RNAP II was localized and recruited into DNA replication compartments after HSV-1 infection.

A series of control experiments, described in Chapter 2, were performed. Mock infected cells showed no detectable staining with 3-83. There was also no cross reaction of the RNAP II and ICP8 antibodies and of the two fluorescence secondary antibodies (data not shown). ICP8 showed some peripheral staining in the nucleus and also in the cytoplasm (Fig. 3.2B and 3.2D), which did not colocalize with RNAP II staining (Fig. 3.2A and 3.2C). These staining patterns for 3-83 and other rabbit polyclonal antisera have been observed previously. Staining along the periphery of the nucleus and in the cytoplasm may be due to a specific interaction between rabbit immunoglobulins and an Fc receptor encoded by HSV-1

(Rice, Unpublished data; Johnson et al., 1988). This particular staining provided us with an internal control for the lack of cross-reaction between the two primary antibodies. Immunofluorescence localization of another abundant nuclear protein, ICP27, showed a distribution throughout the nucleus, confirming that not all nuclear proteins specifically localize to DNA replication compartments (data not shown).

Double immunofluorescence experiments as described above were also performed with HeLa (S3 or CCL2.2) and HEL 299 cells to determine if the localization of RNAP II into viral DNA replication compartments after HSV-1 infection was specific to only Vero cells or was a general phenomenon. The staining pattern of RNAP II after HSV-1 infection in HeLa (S3 or CCL2.2) and HEL 299 cells was found to be similar to that observed in Vero cells (data not shown). In addition, RNAP II colocalized with ICP8, indicating that RNAP II was recruited into DNA replication compartments after infection. Therefore, localization of RNAP II into DNA replication compartments after HSV-1 infection was not restricted to monkey cells but also occurs in human cells.

These data demonstrate that after HSV-1 infection in Vero, HeLa (S3 and CCL2.2), and HEL 299 cells, RNAP II and ICP8 colocalize into viral DNA replication compartments. Also, the differences in the intensities of 8WG16 staining before and after infection suggest that changes in CTD phosphorylation may accompany this localization.

C. RNAP II large subunit is modified following HSV-1 infection

To determine whether HSV-1 infection resulted in any significant modifications to RNAP II, we analyzed the large subunit of RNAP II by Western blotting. Vero cells were mock infected or infected with HSV-1 (KOS1.1) for various times. The cells were then harvested and lysed into Laemmli buffer. Extracts were analyzed by SDS-PAGE and Western blotting, using either the ARNA3 (Fig. 3.3A) or 8WG16 (Fig. 3.3B) monoclonal antibodies.

In normal cells, there is a mixture of two forms of RNAP II differing in only the migration of the large subunit. The IIa form migrates at approximately 200 kDa on SDS-PAGE gels and contains the nonphosphorylated form of the CTD. The IIo form migrates at approximately 240 kDa on SDS-PAGE gels and contains the fully phosphorylated form of the CTD (Kim and Dahmus, 1986; Cadena and Dahmus, 1987; Zhang and Corden, 1991b). The ratio of IIa to IIo varies from cell type to cell type (Bartholmew et al., 1986; Kim and Dahmus, 1988; Lee and Greenleaf, 1989; Kolodziej et al., 1990) and is dramatically altered in response to heat shock (Dubois et al., 1994a), serum induction (Dubois et al., 1994b), and poliovirus infection (Rangel et al., 1987; Rangel et al., 1988). In poliovirus infection, HeLa S3 cells contain a decrease in abundance of total and chromatin bound RNAP IIO. The loss of IIO requires poliovirus gene expression (Rangel et al., 1987; Rangel et al., 1988).

Western blot analysis showed that mock infected Vero cells contained a 1:1 ratio of IIa to IIo (Fig 3.3A, lane 2). The IIa form comigrated with a 200 kDa molecular weight marker (data not shown) and with the IIa form present in a nuclear extract from HeLa S3 cells (Fig 3.3A, lane 1). Nuclear extracts prepared from HeLa S3 cells contained predominantly the IIa form of the large subunit. When the mock infected extracts were probed with 8WG16, only the IIa form was apparent (Fig. 3.3B, lane 2). This was expected as 8WG16 does not react with fully phosphorylated forms of RNAP II large subunit. Infection with HSV-1 resulted in a striking alteration to the RNAP II large subunit. As infection proceeded, there was a loss of IIa and IIo and the appearance of intermediately migrating material, between approximately 215 kDa and 230 kDa (Fig. 3.3A and 3.3B, lanes 5 to 8) on SDS-PAGE gels. This polydisperse species was designated as IIi (or intermediately migrating). The IIi form was observed as early as 3 hr post-infection and became the predominant form by 5 hr post-infection (Fig. 3.3A and 3.3B, lanes 5 and 6). All forms of RNAP II appeared to decrease in abundance at later times (8 hr to 12 hr) in infection as detected by ARNA3 (Fig. 3.3A, lanes 7 and 8). We have not further investigated the reasons for this apparent loss of protein observed after infection. Intermediately migrating forms may be present in high abundance between 215 kDa and 230 kDa, but not readily detected with ARNA3. Because 8WG16 reacts strongly with the IIi form, the protein abundance of RNAP II large subunit does not appear to decline at late times in infection as observed with ARNA3. Since 8WG16 reacts with IIi and IIa and does not react with the fully phosphorylated IIo form, it is possible that the IIi species contains a CTD that is only partially phosphorylated. The strong reactivity of 8WG16 to the IIi species in Western blotting is consistent with the increased immunofluorescence staining that is observed with this antibody after HSV-1 infection (Fig. 3.1B and 3.1D).

We then wanted to determine whether RNAP II modifications were specific to Vero cells and if IIi was formed after HSV-1 infection of human cells. Western blotting experiments using ARNA3 or 8WG16 were performed with HeLa S3 (Fig. 3.4A and 3.4B) and HEL 299 (Fig. 3.5A and 3.5B) extracts infected with HSV-1. Similar results to that observed in infected Vero cells were observed in infected HeLa S3 and HEL 299 cells. IIa and IIo were depleted and IIi was formed. With the ARNA3 antibody, there was an apparent depletion in all forms of RNAP II late in infection. These results indicate that the induction of IIi after HSV-1 infection is not restricted to Vero cells but also occurs in human cell lines such as HeLa S3 and HEL 299 cells.

To ensure that the cells were efficiently infected with HSV-1, a series of control experiments were performed. The same Vero, HeLa S3 and HEL 299 extracts analyzed in Fig. 3.3, 3.4 and 3.5 were analyzed for the presence of specific viral proteins. Vero extracts, mock infected or infected with HSV-1 (KOS1.1), were analyzed on SDS-PAGE gels and Western blotting. The blots were probed with H1101 (specific for ICP4) (Fig. 3.6A), H1113 (specific for ICP27) (Fig. 3.6B) or H1115 (specific for ICP8) (Fig. 3.6C). ICP4, ICP27, and ICP8 were detected in Vero infected cells at 5 hr and 8 hr post-infection but not in mock infected Vero cells. The levels of the IE proteins ICP4 and ICP27 were similar at 5 and 8 hr post-infection (Fig. 3.6A and 3.6B, lanes 2 and 3). Multiple bands were observed for the ICP27 protein, bands which may represent phosphorylation variants or degradation products of ICP27. The levels of ICP8, a DE gene product, increased from 5 to 8 hr post-infection (Fig. 3.6C, lanes 2 and 3). ICP0, ICP4, and ICP27 were detected in HeLa S3 infected cells at 3 hr, 5 hr, and 8 hr post-infection but not in mock infected HeLa S3 cells or in nuclear extract prepared from uninfected HeLa S3 cells (data not shown). ICP27 and ICP8 were detected in HEL 299 infected cell extracts but not in mock infected HEL 299 cells at 3 hr, 6 hr, and 9 hr post-infection (Fig. 3.7A and 3.7B). The levels of ICP27 protein increased from 3 hr to 6 hr post-infection but became somewhat less abundant at 9 hr post-infection (Fig. 3.7A, lanes 2, 4, and 6). As infection progressed, ICP8 protein levels and proteolytic fragments increased (Fig. 3.7B, lanes 2, 4, and 6). These data confirm that the Vero, HeLa S3, and HEL 299 cells were efficiently infected, as various viral IE and DE were synthesized appropriately.

D. RNAP II is aberrantly phosphorylated following HSV-1 infection

Because modifications to the RNAP II large subunit occur exclusively through phosphorylation of the CTD (Dahmus, 1994; Kang and Dahmus, 1995; Dahmus, 1996), we hypothesized that HSV-1 infection may have caused rapid and aberrant phosphorylation of the CTD. An alternative explanation could be that HSV-1 infection resulted in proteolytic cleavage or degradation of the large subunit of RNAP II, thereby generating IIi.

To confirm the identity of the IIi variant, mock infected or WT HSV-1 infected Vero cell extracts were treated with calf intestinal alkaline phosphatase (CIAP). CIAP treated extracts were analyzed by Western blotting and probing with a mixture of 8WG16 and ARNA3. CIAP treatment effectively converted both IIo and IIi to IIa (Fig. 3.8). The data confirm previous CIAP experiments, demonstrating the conversion of IIo to IIa (Cadena and Dahmus, 1987; Lu et al., 1991). The data also confirm that the IIi form is a phosphorylation variant and not a degradation product of the large subunit of RNAP II.

E. The IIo and IIi subunits are phosphorylated on serine and threonine residues

To determine which amino acid residues were phosphorylated in the IIo and IIi forms, we analyzed the IIo and IIi subunits by phosphoamino acid analysis. Vero cells were mock infected or infected with WT HSV-1 and then labeled with ^{32}P-orthophosphate for 5 hr. Nuclear lysates were prepared and RNAP II was immunoprecipitated with ARNA3 or 8WG16. Immunoprecipitates were separated on SDS-PAGE gels and IIo or IIi subunit was isolated and digested with 6 N HCl at 110°C for various periods of time. Liberated amino acids were separated by two dimensional thin layer electrophoresis. Different hydrolysis times in 6 N HCl at 110°C are optimal for the recovery of specific residues from the protein, because the stability of phosphoamino acids to acid treatment is in the order phosphothreonine > phosphoserine > phosphotyrosine (Duclos et al., 1991). In general, the optimal time for phosphoprotein hydrolysis, to allow complete polypeptide hydrolysis with only minimal loss of labile phosphoamino acids, is 2 hr to 3 hr in 6 N HCl at 110°C. Shorter times of hydrolysis (1 hr) in 6 N HCl at 110°C increase the yield of phosphotyrosine relative to phosphoserine and phosphothreonine. Treatment of the protein for longer periods of time (3 hr and 5 hr, in 6 N HCl at 110°C) favors the recovery of phosphoserine and phosphothreonine respectively (Cooper et al., 1983; Duclos et al., 1991).

The IIo form in mock infected cells contained both phosphoserine and phosphothreonine (Fig. 3.9). Likewise, the IIi form in WT HSV-1 (KOS1.1) infected cells contained phosphoserine and phosphothreonine (Fig. 3.10). No phosphotyrosine was detected in either the IIo or IIi subunit. PhosphorImage analysis revealed that at 3 hr in 6 N HCl at 110°C, IIo contained 89% phosphoserine and 11% phosphothreonine (Fig. 3.9). IIi contained 85% phosphoserine and 15% phosphothreonine (Fig. 3.10). Therefore, there was no apparent difference in the type or ratio of phosphorylation (on serine and threonine) between the IIo and IIi subunits.

Our experiments demonstrate that the IIo form is phosphorylated on serine and threonine but not on tyrosine residues. It has been reported that IIo is phosphorylated on serine, threonine and tyrosine residues (Baskaran et al., 1993; Dahmus, 1994; Kang and Dahmus, 1995; Dahmus, 1996). The discrepancy between our findings and those of Baskaran et al. (1993), may be due to a variety of reasons. Despite similar types and concentrations of phosphatase and protease inhibitors used and similar methods performed for nuclear lysate preparations and immunoprecipitations, it is possible that under our experimental conditions, phosphotyrosine was not detectable due to its lability. Baskaran et al. (1993), used the 8WG16 antibody in their immunoprecipitations of RNAP II. We have also used

8WG16 in immunoprecipitations of the ^{32}P-labeled IIi subunit (Fig. 3.10). We did not attempt to immunoprecipitate the ^{32}P-labeled IIo subunit with 8WG16 as the fully phosphorylated form of RNAP II is not recognized by the antibody (Fig. 3.3, 3.4, and 3.5). Baskaran et al. (1993) lysed their nuclei with sonication, rather than with DNase and RNase treatment. Our DNase and RNase treatment of nuclei requires 30 min, whereas sonication requires only several min. It is possible that tyrosine phosphatases have increased opportunities to dephosphorylate the RNAP II large subunit during periods of DNase and RNase treatment but not during sonication. It is also possible that tyrosine phosphorylation or dephosphorylation of RNAP II may be cell type specific. Baskaran et al. (1993) examined the phosphoamino acid composition of RNAP IIO recovered from HeLa S3 cells, whereas we have analyzed the RNAP II large subunit from Vero cells. HeLa S3 and Vero cells may differ in their tyrosine kinase or phosphatase activities.

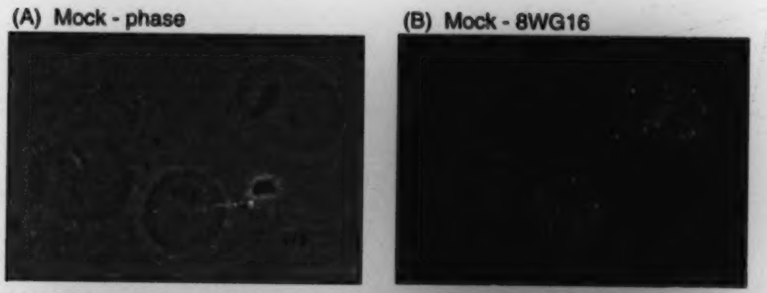
Previous studies using cyanogen bromide to cleave the CTD from the body have shown that all phosphorylation occurs on the CTD and not on the body of the RNAP II large subunit (Cadena and Dahmus, 1987). We were unable to determine if phosphorylation occurred on the CTD or the body of the IIi subunit due to technical difficulties. There was either incomplete cleavage of the CTD from the body of the RNAP II large subunit or a total loss of protein after cyanogen bromide treatment. Thus, we were unable to conclusively determine that phosphorylation of IIi was restricted to the CTD.

F. Summary

After HSV-1 infection of Vero, HeLa, or HEL 299 cells, the RNAP II large subunit is aberrantly modified and relocalized into nuclear viral compartments. The IIa and IIo forms are replaced with the intermediately migrating IIi form, which is a phosphorylation variant of the large subunit. Phosphoamino acids analyses indicate that both IIo and IIi subunits are phosphorylated on serine and threonine residues in similar proportions.

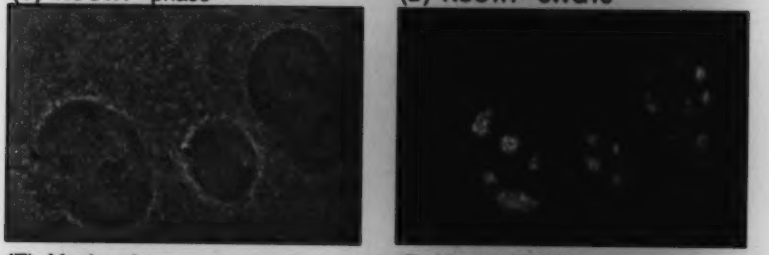
Figure 3.1. Nuclear localization of RNAP II. Vero cells were mock infected (panels A, B, E, and F) or infected with HSV-1 (KOS1.1) (panels C, D, G, and H) for 5 hr. Cells were fixed and stained with monoclonal antibody 8WG16 (panels A to D) or monoclonal antibody ARNA3 (panels E to H). Secondary antibody was rhodamine-conjugated goat anti-mouse immunoglobulin. Phase contrast microscopy (panels A, C, E, and G). Fluorescence microscopy (panels B, D, F, and H). Bar = 10 µm.

Immunofluorescence



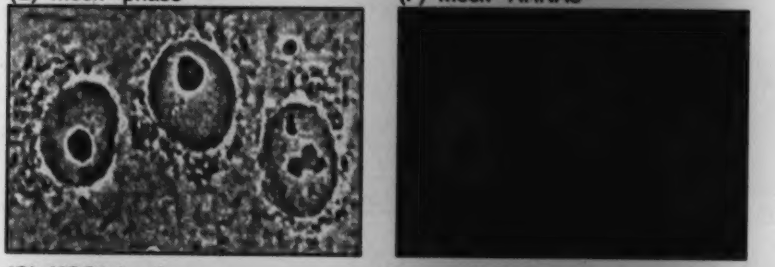
(A) Mock - phase

(B) Mock - 8WG16



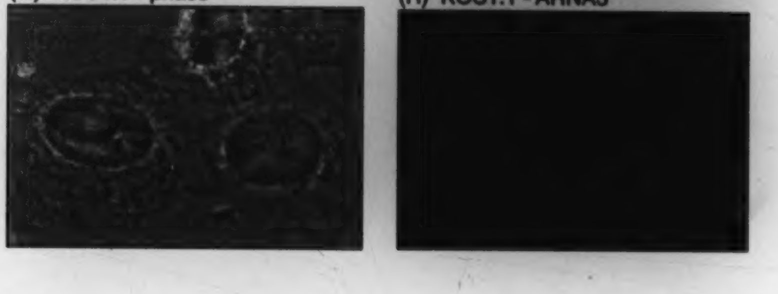
(C) KOS1.1 - phase

(D) KOS1.1 - 8WG16



(E) Mock - phase

(F) Mock - ARNA3

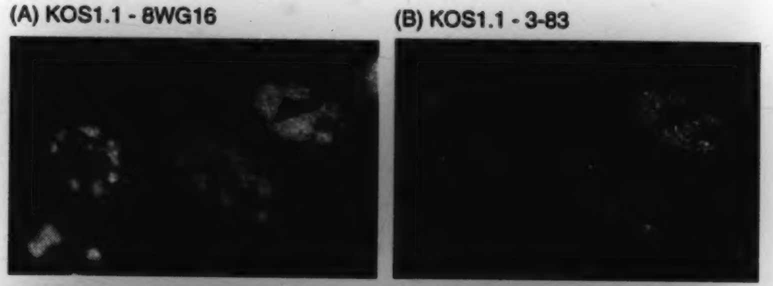


(G) KOS1.1 - phase

(H) KOS1.1 - ARNA3

Figure 3.2. Double immunofluorescence of RNAP II and ICP8 in HSV-1 (KOS1.1) infected Vero cells. Vero cells were infected with HSV-1 (KOS1.1) for 5 hr, fixed and stained with monoclonal antibody 8WG16 and anti-ICP8 polyclonal antiserum 3-83 (panels A and B) or monoclonal antibody ARNA3 and anti-ICP8 polyclonal antiserum 3-83 (panels C and D). Secondary antibodies were a mixture of rhodamine-conjugated goat anti-mouse immunoglobulin (for RNAP II visualization) and fluorescein conjugated donkey anti-rabbit immunoglobulin (for ICP8 visualization). Detection of RNAP II (panels A and C). Detection of ICP8 (panels B and D). Bar = 10 μm.

Double Immunofluorescence



(A) KOS1.1 - 8WG16

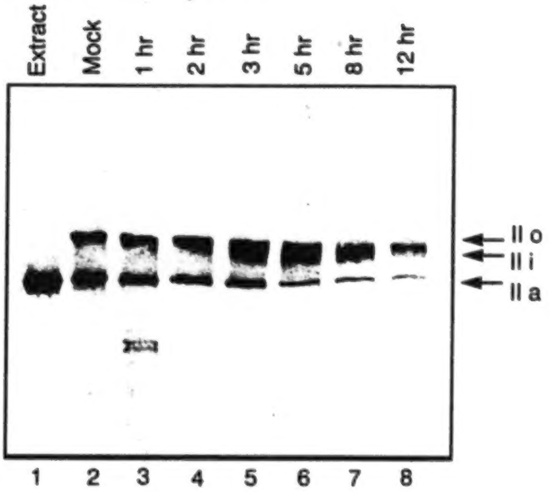
(B) KOS1.1 - 3-83

(C) KOS1.1 - ARNA3

(D) KOS1.1 - 3-83

Figure 3.3. Western blot analysis of the RNAP II large subunit in HSV-1 (KOS1.1) infected Vero cells. Vero cells were mock infected (lanes 2) or infected with HSV-1 (KOS1.1) (lanes 3 to 8) for the times indicated. Cells were harvested and lysed into Laemmli buffer. Whole cell extracts were run on SDS-PAGE (6% acrylamide) and then subjected to immunoblotting. The samples were probed with either monoclonal antibody ARNA3 (A), which reacts with the body of the RNAP II large subunit, or monoclonal antibody 8WG16 (B), which reacts with the CTD of the RNAP II large subunit. Each lane contained extract from the same number of cells, except lane 1, which contained an aliquot of a nuclear *in vitro* transcription extract prepared from HeLa cells. Subunit IIa migrates at approximately 200 kDa; IIo migrates at approximately 240 kDa.

A. Western - ARNA3


Extract
Mock
1 hr
2 hr
3 hr
5 hr
8 hr
12 hr
II o
II i
II a
1
2
3
4
5
6
7
8

B. Western - 8WG16

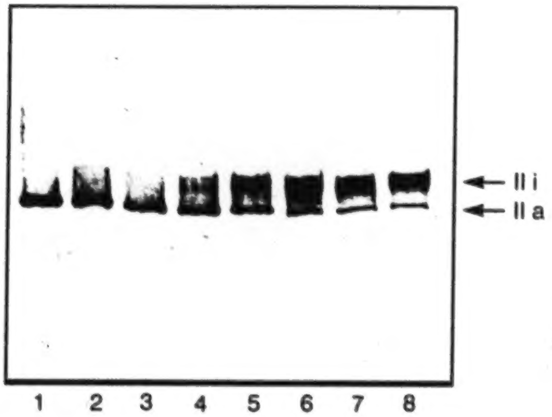
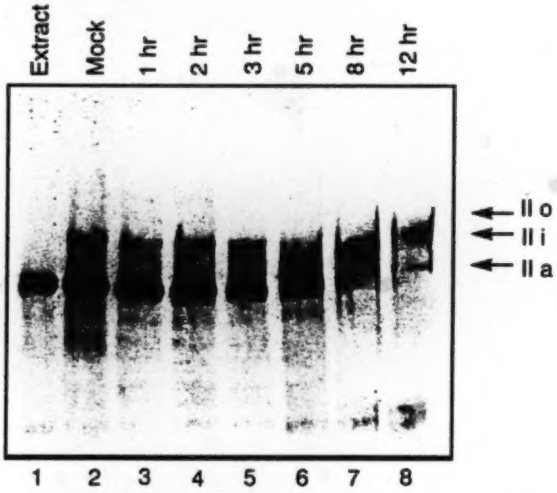

II i
II a
1
2
3
4
5
6
7
8

Figure 3.4. Western blot analysis of the RNAP II large subunit in HSV-1 (KOS1.1) infected HeLa S3 cells. HeLa S3 cells were mock infected (lanes 2) or infected with HSV-1 (KOS1.1) (lanes 3 to 8) for the times indicated. Samples were prepared and analysis was done as described in the legend of Fig. 3.3. Each lane contained extract from the same number of cells, except lane 1, which contained an aliquot of a nuclear *in vitro* transcription extract prepared from HeLa cells. We have observed that HeLa nuclear *in vitro* transcription extracts characteristically contain predominantly the IIa form of the RNAP II large subunit, in contrast to the mixtures of IIa and IIo that are observed when the same cells are lysed directly into Laemmli buffer (lanes 1 versus lanes 2). Because the IIO form of RNAP II is the form involved in transcription elongation and the IIA form is the form that enters the preinitiation complex (Dahmus, 1994; Kang and Dahmus, 1995; Dahmus, 1996), IIA may be more soluble and preferentially extracted during nuclear extractions. Alternatively, IIA may become dephosphorylated during nuclear extractions.

A. Western - ARNA3


Extract
Mock
1 hr
2 hr
3 hr
5 hr
8 hr
12 hr
II o
II i
II a
1
2
3
4
5
6
7
8

B. Western - 8WG16

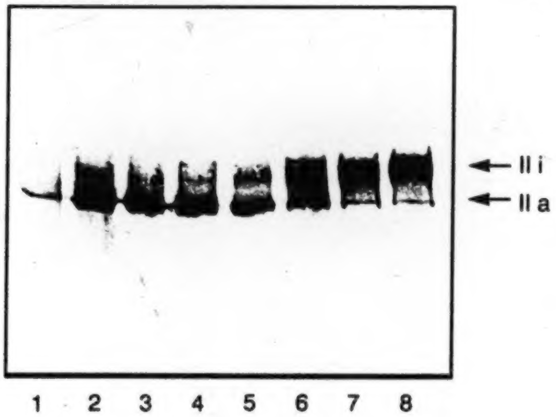
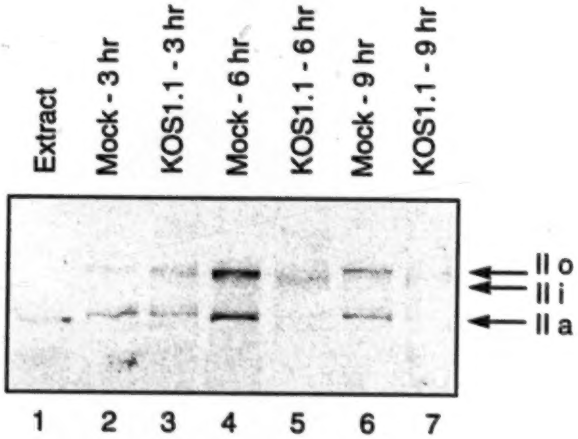

II i
II a
1
2
3
4
5
6
7
8

Figure 3.5. Western blot analysis of the RNAP II large subunit in HSV-1 (KOS1.1) infected HEL 299 cells. HEL 299 cells were mock infected (lanes 2, 4, and 6) or infected with HSV-1 (KOS1.1) (lanes 3, 5, and 7) for the times indicated. Samples were prepared and analysis was conducted as described in the legend of Fig. 3.3. Each lane contained extract from the same number of cells, except lane 1, which contained an aliquot of a nuclear *in vitro* transcription extract prepared from HeLa cells. The blots were probed with RNAP II large subunit antibodies ARNA3 (A) or 8WG16 (B).

A. Western - HEL- ARNA3


Extract
Mock - 3 hr
KOS1.1 - 3 hr
Mock - 6 hr
KOS1.1 - 6 hr
Mock - 9 hr
KOS1.1 - 9 hr
II o
II i
II a
1
2
3
4
5
6
7

B. Western - HEL- 8WG16

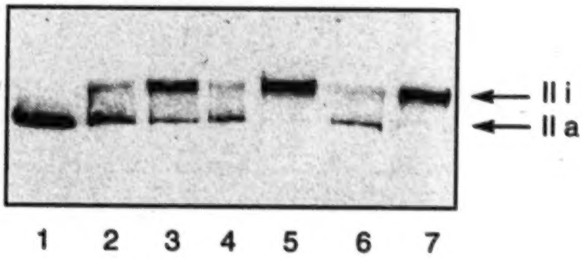
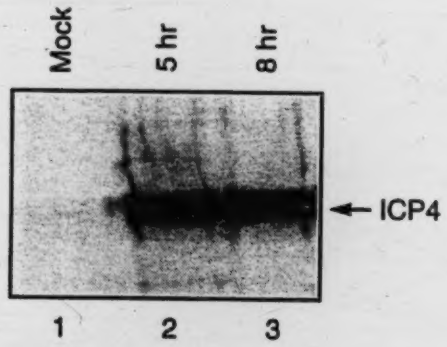
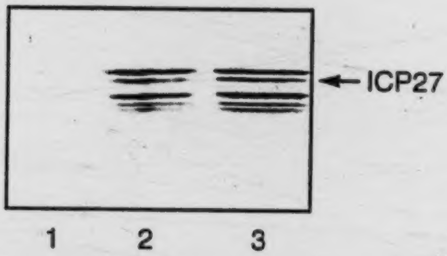

II i
II a
1
2
3
4
5
6
7

Figure 3.6. Western blot analysis of specific viral proteins from HSV-1 (KOS1.1) infected Vero cells. Vero mock infected (lanes 1) and HSV-1 (KOS1.1) infected (lanes 2 and 3) samples analyzed in Fig. 3.3 were run on SDS-PAGE (6% [A and C] or 12% [B] acrylamide) gels and immunoblotted. The samples were probed with either monoclonal antibody H1101 (A), which recognizes ICP4, monoclonal antibody H1113 (B), which recognizes ICP27, or monoclonal antibody H1115 (C), which recognizes ICP8. ICP4, ICP27 and ICP8 migrate at approximately 175 kDa, 63 kDa and 120 kDa respectively.

A. Western - Vero - ICP4


Mock
5 hr
8 hr
ICP4
1
2
3

B. Western - Vero - ICP27



C. Western - Vero - ICP8

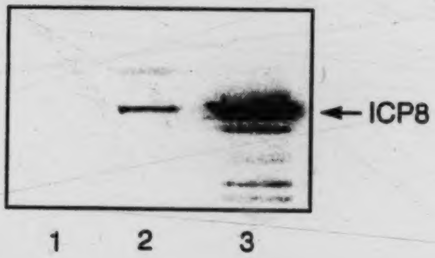
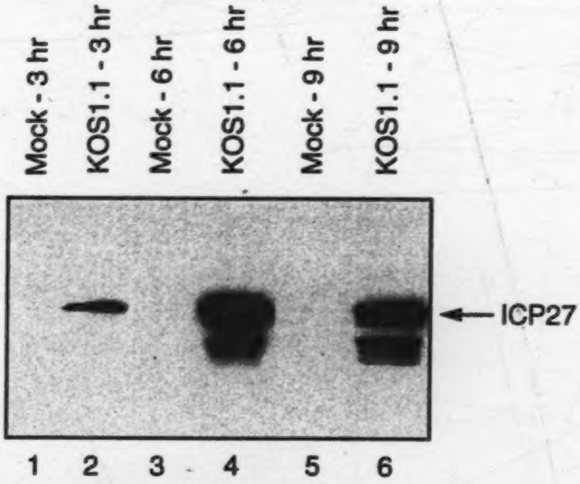
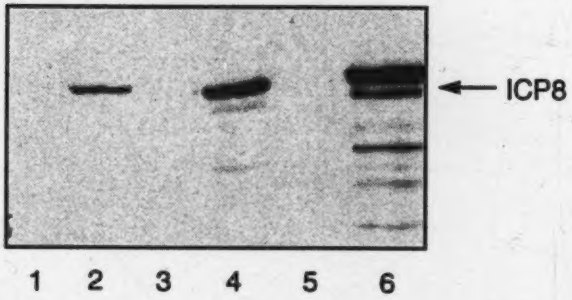


Figure 3.7. Western blot analysis of specific viral proteins from HSV-1 (KOS1.1) infected HEL 299 cells. HEL 299 mock infected (lanes 1, 3, and 5) and HSV-1 (KOS1.1) infected (lanes 2, 4, and 6) samples analyzed in Fig. 3.5 were run on SDS-PAGE (6% [A] or 12% [B] acrylamide) gels and immunoblotted. The samples were probed with either monoclonal antibody H1113 (A), which recognizes ICP27 or monoclonal antibody H1115 (B), which recognizes ICP8. ICP27 and ICP8 migrate at approximately 63 kDa and 120 kDa respectively.

A. Western - HEL- ICP27


Mock - 3 hr
KOS1.1 - 3 hr
Mock - 6 hr
KOS1.1 - 6 hr
Mock - 9 hr
KOS1.1 - 9 hr
ICP27
1
2
3
4
5
6

B. Western - HEL- ICP8


ICP8
1
2
3
4
5
6

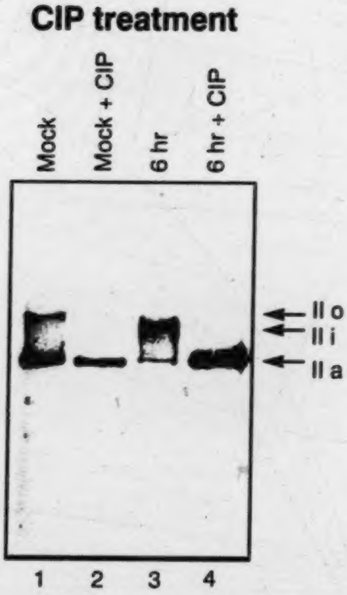
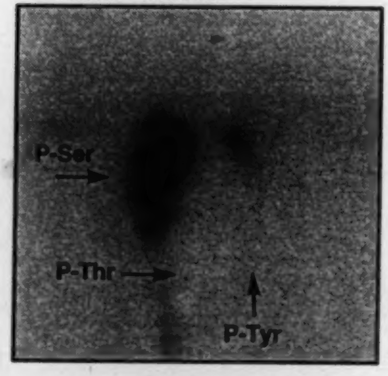


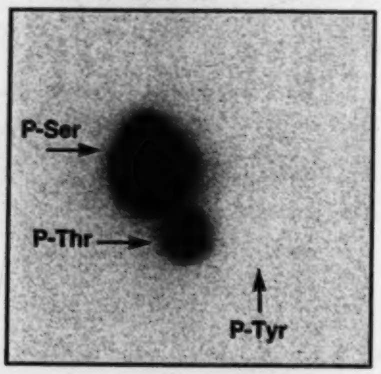
Figure 3.8. Western blot analysis of CIAP treated RNAP II large subunit. Laemmli extracts were prepared from mock infected Vero cells or from Vero cells infected with HSV-1 (KOS1.1) for 6 hr. Whole cell extracts were dialyzed against CIAP compatible buffer to remove SDS and were treated with 50 units of CIAP for 30 min. Treated and untreated extracts were separated by SDS-PAGE (6% acrylamide) and then subjected to immunoblotting. The samples were then probed with a mixture of antibodies ARNA3 and 8WG16. Lanes: 1 and 2, mock infected cell extracts, untreated or treated with CIAP; 3 and 4, KOS1.1 infected cell extracts, untreated or treated with CIAP.

Figure 3.9. Phosphoamino acid analysis of the IIo subunit from mock infected Vero cells. Mock infected Vero cells were labeled with ^{32}P-orthophosphate for 5 hr. Nuclear lysates were prepared and the large subunit of RNAP II was immunoprecipitated with ARNA3. Immunoprecipitates were run on SDS-PAGE and blotted onto Immobilon-P membrane. The membrane was subsequently exposed to film in order to visualize and identify the ^{32}P-labeled IIo subunit. The IIo subunit was cut from the membrane and digested with 6 N hydrochloric acid (HCl) at 110°C for 1 (A), 3 (B), or 5 hr (C). Liberated amino acids were separated electrophoretically. The details of these experiments are described in Chapter 2. Different hydrolysis times in HCl are optimal for the recovery of specific amino acids from the protein. The stability of phosphoamino acids to HCl treatment is in the order of phosphothreonine > phosphoserine > phosphotyrosine (Duclos et al., 1991). For maximal yields of phosphotyrosine, phosphoserine, and phosphothreonine, the optimal times of hydrolysis in 6 N HCl at 110°C are 1, 3 and 5 hr respectively (Cooper et al., 1983; Duclos et al., 1991).

A. Phosphoamino Acid Analysis - Mock - 1 hr HCl


P-Ser
P-Thr
P-Tyr

B. Phosphoamino Acid Analysis - Mock - 3 hr HCl


P-Ser
P-Thr
P-Tyr

C. Phosphoamino Acid Analysis - Mock - 5 hr HCl

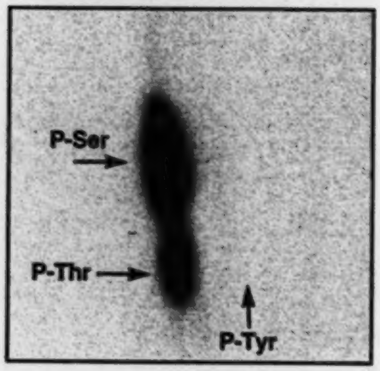
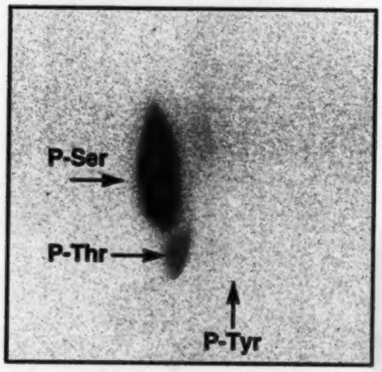
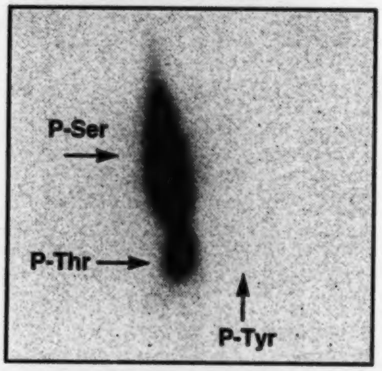

P-Ser
P-Thr
P-Tyr

Figure 3.10. Phosphoamino acid analysis of the IIi subunit from HSV-1 infected Vero cells. Vero cells were infected with WT HSV-1 (KOS1.1) and then analyzed as described in Fig. 3.10, except the IIi subunit (instead of IIo) was examined. The IIi subunit was immunoprecipitated with 8WG16. Panels: A, 1 hr 6 N HCl hydrolysis at 110°C; B, 3 hr 6 N HCl hydrolysis at 110°C; 5 hr 6 N HCl hydrolysis at 110°C.

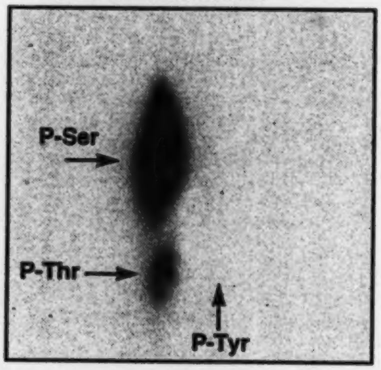
A. Phosphoamino Acid Analysis - WT HSV-1 - 1 hr HCl


P-Ser
P-Thr
P-Tyr

B. Phosphoamino Acid Analysis - WT HSV-1 - 3 hr HCl


P-Ser
P-Thr
P-Tyr

C. Phosphoamino Acid Analysis - WT HSV-1 - 5 hr HCl


P-Ser
P-Thr
P-Tyr

CHAPTER FOUR. MODIFICATIONS TO THE RNAP II LARGE SUBUNIT REQUIRE THE PRESENCE OF THE VIRAL GENE PRODUCT, ICP22 [2, 3]

A. Introduction

Viral transcription is induced and cellular transcription is suppressed during HSV-1 lytic infection (Smiley et. al, 1991; Spencer et al., 1997). The transcription of viral genes is dependent on the host's RNAP II transcription machinery. The question of how the virus facilitates this shift in RNAP II transcription is still unresolved.

Recent evidence suggests that the shift from cellular to viral transcription involves sequence-independent mechanisms such as modifications to the RNAP II transcription apparatus, alterations in DNA conformation and localization of transcription proteins and viral genes into specialized nuclear compartments (Smiley et al., 1991). Consistent with this hypothesis, we have found that HSV-1 infection results in dramatic modifications to RNAP II. Firstly, RNAP II is recruited from a diffuse nuclear distribution into viral DNA replication compartments after infection. Secondly, the large subunit of RNAP II is rapidly and aberrantly phosphorylated, most likely on its CTD. The IIo and IIa forms are depleted and the IIi form is induced (Chapter 3). In this chapter, I report that RNAP II modifications require new viral protein synthesis and specifically the expression of the IE protein ICP22.

A version of this chapter has been published:

[2] Rice, S. A., M. C. Long, V. Lam, and C. A. Spencer. 1994. RNA polymerase II is aberrantly phosphorylated and localized to viral replication compartments following herpes simplex virus infections. J. Virol. 68: 988-1001.

[3] Rice, S. A., M. C. Long, V. Lam, P. A. Schaffer, and C. A. Spencer. 1995. Herpes simplex virus immediate-early protein ICP22 is required for viral modification of host RNA polymerase II and establishment of the normal viral transcription program. J. Virol. 69:5550-5559.

B. RNAP II modifications occur in the absence of viral DNA replication and recruitment into replication compartments

The conversion of the IIo form to the IIi form occurs between 2 and 5 hr post-infection, concomitant with the formation of DNA replication compartments (Gao and Knipe, 1989) and the recruitment of RNAP II into these compartments (Chapter 3). To determine whether DNA replication compartment formation and viral DNA replication are requirements for RNAP II localization after infection and to determine the relationship between subnuclear localization and RNAP II phosphorylation patterns, we analyzed RNAP II large subunit modification and localization in Vero cells infected with the ICP8 mutant virus, *d*101 (Gao and Knipe, 1989). The ICP8 mutant virus contains a deletion in the gene encoding ICP8, a DE protein essential for HSV-1 DNA replication and for the formation of viral replication compartments. Cells infected with *d*101 express IE and DE proteins, with the exception of ICP8, but are arrested prior to DNA replication (Gao and Knipe, 1989).

Vero cells were infected with *d*101 (Gao and Knipe, 1989) for 5 hr, fixed and stained with primary RNAP II large subunit antibody, 8WG16, and secondary fluorescence antibody, GAMR (Fig. 4.1B). The RNAP II large subunit displayed a diffuse nuclear distribution with some punctate concentrations, representing prereplicative structures as observed previously (Quinlan et al., 1984; de Bruyn Kops and Knipe, 1988). RNAP II modifications were examined by Western blotting, using RNAP II antibodies 8WG16 and ARNA3 and extracts prepared from Vero cells infected with either WT HSV-1 (KOS1.1) or *d*101. Despite the absence of both DNA replication compartments and the recruitment of RNAP II into replication compartments, modifications to RNAP II occurred (Fig. 4.1A and 4.1B) as in KOS1.1 infection (Fig. 3.1, 3.2, and 3.3). The IIi subunit was produced in *d*101 infected cells to similar degrees and at similar times as in KOS1.1 infected cells. These experiments were also conducted with *d*101 infected HeLa S3 and HEL 299 extracts and similar results were obtained (data not shown).

From these data, we conclude that neither DNA replication nor the formation of viral replication compartments is necessary for aberrant phosphorylation of the RNAP II large subunit. In addition, neither ICP8 activity nor the expression of true L (γ2) gene products, whose appearance is dependent on viral DNA replication, is required for RNAP II modifications. Although RNAP II recruitment into DNA replication compartments is not required for RNAP II large subunit modifications, our data do not address whether RNAP II modifications are a prerequisite for recruitment into these compartments.

C. RNAP II modifications require early expression of the viral genome

Having established that modifications to RNAP II occur early in infection and are not dependent on viral DNA replication, ICP8 activity, or DNA replication dependent L proteins, we sought to determine which viral gene products or activities were responsible for RNAP II modifications. Two general hypotheses are possible. Firstly, the conversion to the IIi subunit could result from the function of a virion component, such as VP16. Secondly, IE and DE gene products could be required for RNAP II modifications.

To determine if RNAP II modifications were dependent on virion components alone, we performed UV-inactivation experiments. UV inactivation of whole virus has been used previously to define the relative contributions of virion proteins entering the cell and proteins synthesized early in infection (Fenwick and Clark, 1982; Hill et al., 1983; Hill et al., 1985; Fenwick and Owen, 1988). Treatment of HSV-1 virus with shortwave (254 nm) UV light, under appropriate conditions, crosslinks DNA, thereby preventing transcription of viral genes. Such UV treatment does not damage virion proteins (Hill et al., 1983).

The preparation of UV-inactivated virions and the characterization of virion components after UV-inactivation were performed by Dr. Stephen Rice and Vivian Leong (Lam), University of Alberta, Edmonton, Alberta (Fig. 4.2A). A suspension of WT HSV-1 was treated with 254 nm UV light, which lowered the virus titer by 4 to 5 orders of magnitude. To confirm that UV treatment did not destroy the function of the virion components, the UV-inactivated virions were tested for their ability to specifically transactivate an IE gene promoter. Vero cells were transiently transfected with the plasmid pIE3-CAT (DeLuca et al., 1985), which contains the chloramphenicol acetyltransferase (CAT) gene under the control of the ICP4 gene promoter. The ICP4 promoter is transcriptionally activated by VP16 *cis*-acting response elements and should be stimulated by the presence of virions containing functional VP16. As a control, cells were transiently transfected with the plasmid p8-CAT (Su and Knipe, 1989), which contains the CAT gene under the control of the ICP8 gene promoter. The DE ICP8 gene is not transactivated by VP16, since it does not contain IE response elements. At 24 hr after transfection, identical transfected cells were either mock infected or infected with UV-inactivated virus. At 24 hr post-infection, the cells were harvested, extracts were prepared and CAT assays were performed. The UV-inactivated virions transcriptionally activated the ICP4 promoter, confirming that VP16 was active (Fig. 4.2A, lanes 1 and 2), indicating that virion components remain active after UV treatment. As expected, the ICP8 promoter was not significantly transactivated by the UV-inactivated virions (Fig. 4.2A, lanes 3 and 4). ICP8 transcription is not transactivated by VP16 but instead is stimulated by ICP4 (Roizman and Sears, 1990). Because the ICP8 promoter in transfected cells is not transactivated by UV-inactivated virions, this demonstrates that IE gene expression is efficiently prevented in infected cells.

To determine whether virion components alone are sufficient to trigger modifications to RNAP II, the RNAP II large subunit was analyzed in mock infected Vero cells or Vero cells infected with WT HSV-1 (KOS1.1 or mock UV-inactivated) or UV-inactivated virus. Western blots were probed with antibodies directed against the large subunit of RNAP II, 8WG16 and ARNA3 (Fig. 4.2B). Infection with mock UV-inactivated virions resulted in a loss of the IIa and IIo forms and the appearance of the IIi form (Fig. 4.2B, lane 2). However, infection with the UV-inactivated virus produced no modifications to RNAP II large subunit (Fig. 4.2B, lane 3) and was similar to what was observed in an extract of mock infected Vero cells (Fig. 4.2B, lane 1). These results suggest that virion components, such as VP16, are not in themselves sufficient to trigger modifications to RNAP II large subunit and that subsequent viral gene expression is required.

To confirm that new protein synthesis, and not just viral gene transcription, is required for RNAP II modifications, experiments using protein synthesis inhibitors were performed. Cycloheximide has been used extensively in studies examining the contributions of IE gene expression to the virus life cycle (Honess and Roizman, 1974; Kwong and Frenkel, 1987; Fenwick and Owen, 1988). Infection of cells in the presence of cycloheximide allows the transcription of IE mRNAs but prevents their translation. In the presence of cycloheximide, DE and L genes are not expressed, because their transcription is dependent on the activation functions of the IE proteins, especially ICP4. When cycloheximide is washed out, translation of accumulated IE mRNAs proceeds immediately and DE and L genes are then expressed (Honess and Roizman, 1974; Mackem and Roizman, 1981). In our experiments, Vero cells were treated with cycloheximide and then either mock infected or infected with HSV-1 for 6 hr in the continued presence of cycloheximide. The RNAP II large subunit in whole cell extracts was analyzed by Western blotting using 8WG16 and ARNA3. Cycloheximide treatment prevented modifications to the RNAP II large subunit (Fig. 4.3, lane 5). When cycloheximide was washed out and the cells were incubated for an additional 2 hr at 37°C (Fig. 4.3, lane 6), the RNAP II large subunit was modified to a similar extent as that observed in untreated but HSV-1 infected cells (Fig. 4.3, lane 7). Western blotting was done with the above samples and blots were probed with antibodies directed against viral proteins such as ICP0, ICP4, ICP27, and ICP8 (data not shown). These experiments verified that viral protein synthesis was fully inhibited during cycloheximide treatment but proceeded after washout.

One of the first effects of HSV-1 infection is an efficient shutoff of most of the host cell's protein synthesis (Sydiskis and Roizman, 1966; Hill et al., 1985; Mayman and Nishioka, 1985). If normal phosphorylation of the large subunit of RNAP II is dependent on the presence of a labile host protein, then virus induced shutoff of host cell protein synthesis could be responsible for the modifications to RNAP II large subunit that we observe. However, the inhibition of protein synthesis with cycloheximide in mock infected Vero cells did not alter RNAP II phosphorylation patterns (Fig. 4.3, lanes 2 and 3). We have also seen no modifications to the RNAP II large subunit after treatment of mock infected Vero cells with another translation inhibitor, anisomycin (data not shown). Furthermore, no RNAP II modifications were observed in the UV-inactivation experiment (Fig. 4.3B), where virion-mediated protein synthesis shutoff would have occurred (Schek and Bachenheimer, 1985). Therefore, RNAP II modifications require expression of the viral genome and are not effected solely through the actions of cellular or virion associated proteins.

Because the IIi form is not induced in a UV-inactivated virus infection or in the presence of protein synthesis inhibitors (cycloheximide), one or more newly synthesized viral gene products may be required for modifications to RNAP II. Modifications are effected rapidly, within 2 hr of the appearance of viral proteins. Furthermore, the IIi subunit is induced in the absence of ICP8 activity, DNA replication and true L (γ2) gene expression. These results suggest that the expression of one or more HSV-1 IE or DE proteins (other than ICP8) is required for RNAP II modifications.

A classic method for separating the effects of IE proteins from DE proteins during HSV-1 infection is a cycloheximide reversal in the presence of actinomycin D (Honess and Roizman, 1974). When actinomycin D, a transcription inhibitor, is added during a cycloheximide washout, synthesis of IE proteins occurs but DE and L genes are not transcribed. It is thus possible to differentiate between the effects of the gene products of one temporal gene class from another. We found that treatment of mock infected or HSV-1 infected cells with actinomycin D resulted in a depletion of all forms of the RNAP II large subunit and the appearance of an intermediately migrating form of the large subunit, which migrated between the IIo and IIi positions (data not shown). Another transcription inhibitor, 5, 6-dichlorobenzimidazole riboside (DRB), had similar effects on the RNAP II large subunit (data not shown). These results precluded the use of actinomycin D or DRB reversal experiments to determine the effects of IE versus DE proteins on RNAP II modifications. The effects of actinomycin D and DRB on RNAP II phosphorylation suggest that normal phosphorylation of the RNAP II large subunit may require continued transcription activity. The depletion and apparent phosphorylation changes in the RNAP II large subunit during treatment with transcription inhibitors such as actinomycin D and DRB are not entirely surprising. When transcription is inhibited, RNAP IIA and RNAP IIO are no longer involved in active transcription and may be susceptible to either degradation or dephosphorylation.

D. HSV-1 induced modifications to RNAP II do not require ICP4

The preceding experiments demonstrate that viral protein synthesis is required for the induction of the IIi subunit and that the expression of one or more of HSV-1 IE or DE proteins (other than ICP8 and true L (γ2) proteins dependent on ICP8 for their expression) are required for RNAP II modifications. To identify which IE and/or DE gene products are involved in IIi induction, we first tested whether the IE protein, ICP4, which is required for transcription of DE and L genes (Preston, 1979; Watson and Clements, 1980; DeLuca et al., 1984; DeLuca and Schaffer, 1985; Godowski and Knipe, 1986), was necessary for the formation of the IIi form. Cells were infected with the HSV-1 ICP4 deletion mutant, *d*120, which encodes a severely truncated ICP4 incapable of transcriptional activation (DeLuca et al., 1985). Cell extracts from WT HSV-1 (KOS1.1) or *d*120 infected Vero cells were prepared at 3, 6, and 9 hr post-infection. The proteins were separated on SDS-PAGE gels and immunoblotting was done using antibodies directed against the RNAP II large subunit, 8WG16 and ARNA3. IIi was efficiently induced in *d*120 infected cells (Fig. 4.4, lanes 6 to 8) with kinetics that were comparable to those in WT HSV-1 (KOS1.1) infected cells (Fig. 4.4, lanes 3 to 5). Therefore, the expression of ICP4 is not required for the formation of the IIi subunit. Furthermore, DE and L gene products, which are dependent on ICP4 for their expression are also not required for the induction of the IIi form. These results, together with the results of the UV inactivation and protein synthesis inhibitor studies, suggest that the induction of IIi requires the expression of one or more IE proteins other than ICP4.

E. IIi induction requires the IE protein ICP22

In addition to ICP4, HSV-1 encodes four other IE proteins: ICP0, ICP22, ICP27, and ICP47. HSV-1 also encodes another protein, ICP6, which is generally not classified as an IE protein but is expressed very early in infection and is not dependent on ICP4 for its expression (DeLuca et al., 1985; Desai et al., 1993). Therefore, these four IE proteins and ICP6 are potential factors involved in RNAP II modifications. ICP0, ICP22, and ICP27 are nuclear proteins that play regulatory roles in HSV-1 gene expression. ICP6 is the large subunit of the HSV-1 encoded ribonucleotide reductase and possesses an amino terminal region with autophosphorylating and protein kinase activity (Chung et al., 1989; Conner et al., 1992). ICP47 is a cytosolic protein that inhibits antigen presentation to $CD8^+$ T lymphocytes (York et al., 1994).

To determine whether ICP0, ICP6, ICP27, or ICP22 are required for the induction of IIi, we analyzed RNAP II modifications in Vero cells infected with HSV-1 mutants defective in each of these proteins. The mutants used were (i) *n*212, an ICP0 gene nonsense mutant which expresses 212 amino terminal residues of the 775 residue ICP0 protein and is deficient in ICP0 *trans*-regulatory functions (Cai et al., 1993); (ii) ICP6 , an ICP6 gene deletion mutant (Goldstein and Weller, 1988); (iii) *d*27-1, an ICP27 gene deletion mutant (Rice and Knipe, 1990); and (iv) 22/*n*199, an ICP22 gene nonsense mutant which expresses only 199 amino terminal residues of the 420 residue ICP22 protein (Astor et al., Unpublished data). Cell extracts were prepared after infection at 5 and 10 hr post-infection, and the proteins were separated on SDS-PAGE gels and analyzed by Western blotting. Blots were probed with antibodies directed against the large subunit of RNAP II, ARNA3 (Fig. 4.5A) or 8WG16 (Fig. 4.5B). The ICP0, ICP6, and ICP27 mutants (Fig. 4.5, lanes 4 and 5, 8 and 9, and 10 and 11, respectively) were all similar to the WT HSV-1 virus (Fig. 4.5, lane 3) in their abilities to induce IIi. Also, these mutant infections were similar to the WT infection in that both the IIa and IIo forms were depleted as infection proceeded. In contrast, cells infected with the ICP22 mutant, 22/*n*199 (Fig. 4.5, lanes 6 and 7), showed striking differences from the WT HSV-1 infection at 5 and 10 hr post-infection. After infection with 22/*n*199, IIi was not induced efficiently, although some immunoreactive material, comigrating with IIi, was observed. There was a significant amount of depletion of the IIo subunit after 22/*n*199 infection, but the IIa subunit was retained to a greater degree than after WT HSV-1 infections. A Western blot of these samples probed with an ICP27 antibody, H1113, showed that 22/*n*199 infected cells expressed high levels of ICP27, indicating that the cells had been efficiently infected with HSV-1 (data not shown).

We next analyzed RNAP II modifications in HeLa S3 cells infected with WT and ICP22 mutant viruses. There was a depletion of IIo and IIa and an induction of IIi in HeLa S3 cells infected with WT HSV-1 (KOS1.1) (Fig. 4.6, lanes 6 and 9). At 3 hr post-infection, KOS1.1 and 22/*n*199 infected cells showed little phosphorylation differences from mock infected HeLa S3 cells (Fig. 4.6, lanes 3 and 4 respectively versus lane 2). However, as infection proceeded, at 6 and 9 hr post-infection, 22/*n*199 infected cells showed more similarities with mock infected cells than with KOS1.1 infected cells. In 22/*n*199 infected HeLa S3 cells, there was a retention of both IIa and IIo and little, if any induction of IIi (Fig. 4.6, lanes 7 and 10). This result was slightly different from that in 22/*n*199 infected Vero cells, which showed a depletion of IIo and low but detectable levels of IIi (Fig. 4.5, lanes 6 and 7). The amount of IIo and IIi observed after 22/*n*199 infection in HeLa S3 cells varied from experiment to experiment. In some experiments, very low levels of IIi were produced after 22/*n*199 infection of HeLa S3 cells. What was consistent in all experiments with 22/*n*199 infected HeLa S3 cells was that IIi was not efficiently induced.

All ICP22 mutants show cell specific dependent replication defects. ICP22 mutant viruses grow efficiently in some cell lines, such as Vero cells, but not in others, such as baby hamster kidney cells and HEL cells (Astor et al., Unpublished data; Sears et al., 1985; Poffenberger et al., 1993). To date, it is not known whether HeLa S3 cells are permissive or restrictive for ICP22 mutant infection. It is possible that in permissive cells, host factors are able to complement ICP22 function. As a result, growth is not compromised. In contrast, restrictive cells which lack ICP22 complementing factors show growth impairments.

We were interested in examining whether RNAP II modifications were dependent on ICP22 in a cell line that is restrictive for ICP22 mutant growth. Cellular extracts were prepared from mock infected, HSV-1 (KOS1.1) infected, and 22/*n*199 infected HEL 299 cells and then separated on SDS-PAGE gels. Western blotting was performed and blots were probed with antibodies directed against the large subunit of RNAP II: ARNA3 and 8WG16.

HEL 299 cells infected with WT HSV-1 showed RNAP II phosphorylation changes similar to those observed with Vero and HeLa S3 cells. There was a depletion of the IIo and IIa forms and an induction of the IIi form (Fig. 4.7, lanes 3 and 7). At 4 hr post-infection, 22/*n*199 infection appeared to induce some IIi, but the levels were quite reduced compared with those detected following WT HSV-1 infection (Fig. 4.7, lane 4 versus lane 3). At later times of infection with the 22/*n*199 virus, HEL 299 cells showed efficient depletion of the IIo subunit, and little, if any, formation of IIi (Fig. 4.7, lane 8). This latter result differed somewhat from that seen in Vero cells, where 22/*n*199 appeared to induce low but detectable levels of IIi at late times (Fig. 4.5, lane 7). Although the results differed among three cell lines, it appeared that ICP22 was required in Vero, HeLa S3 and HEL 299 cells for efficient induction of IIi as well as for the depletion of IIa.

In our experiments, 22/*n*199 failed to induce WT levels of IIi in Vero cells, in which they grow well, and in HEL 299 cells, in which they grow poorly. The effects of the ICP22 mutant virus infection on the large subunit of RNAP II did not appear to correlate with the mutant's cell type dependent growth phenotype. However, more subtle phenotypic differences were present in Vero, HeLa S3, and HEL 299 cells following ICP22 mutant infection. Low levels of IIi were detected in 22/*n*199 infected Vero cells (Fig. 4.5A and 4.5B, lanes 7) but were not observed in 22/*n*199 infected HEL 299 cells (Fig. 4.7, lane 8) at late times in infection. In some experiments, HeLa S3 cells also showed detectable levels of IIi following 22/*n*199 infection at late times (Fig. 4.6, lane 10). Therefore, the level of IIi impairment present after 22/*n*199 infection was dependent on the host cell. The presence of complementing host factors in permissive cells may explain why small amounts of IIi are still observed in ICP22 mutant infected Vero cells. Since restrictive cells lack ICP22 complementing factors, IIi is efficiently depleted in ICP22 mutant infected HEL 299 cells.

To ensure that the effect on IIi induction was due to the engineered nonsense mutation in the ICP22 gene and not due to an acquired secondary mutation mapping elsewhere in the 22/*n*199 genome, we analyzed IIi induction in HEL 299 cells infected with 22/*n*199R (Astor et al., Unpublished data), a 22/*n*199 revertant virus. The 22/*n*199R virus was constructed by a marker transfer experiment using 22/*n*199 DNA and a plasmid ICP22 gene fragment. Recombinant viruses containing the ICP22 gene were identified and isolated (Astor et al., Unpublished data). The induction of IIi and the depletion of IIa and IIo in 22/*n*199R infected cells occurred to similar degrees and at similar times as in WT HSV-1 (KOS1.1) infected cells (Fig. 4.7, lanes 5 and 9). We conclude that the observed effects on the RNAP II large subunit during 22/*n*199 infection result from the engineered mutation in the ICP22 gene.

Although 22/*n*199 infection does not induce WT levels of IIi or cause the depletion of IIa, it does result in the depletion of IIo in Vero and HEL 299 cells, suggesting that another viral protein may be involved in this RNAP II modification. IIo depletion occurs efficiently in cells infected with the ICP4 mutant *d*120 but not in cells infected with the UV-inactivated virus. These results suggest that an IE gene product other than ICP4 is required for IIo depletion. However, infection of viruses mutant in the other IE genes, ICP0, ICP6, ICP27, and ICP22 all result in the depletion of the IIo form. It is possible that IE proteins are redundant in their ability to deplete IIo. It will be of interest to determine the phosphorylation patterns of RNAP II large subunit in viruses mutant in more than one IE gene.

Further characterization of specific regions of the ICP22 protein will reveal the residues which are important for RNAP II modification. Interestingly, two differently sized transcripts are produced from the ICP22 domain. The full-length ICP22 protein is encoded by the longer transcript, while a smaller 273 amino acid protein, identical to the C terminus of the full-length ICP22 protein, is encoded by the smaller transcript (amino acids 147 to 420). The shorter ICP22 protein is currently of unknown function (Carter and Roizman, 1996). It would be of interest to determine if the shorter ICP22 protein or the C terminal domain of ICP22 is responsible for RNAP II modifications.

F. Infection with the *d*22 virus affects RNAP II large subunit phosphorylation patterns and IIi induction

Our results show that ICP22 is required for IIi induction in Vero, HeLa S3 and HEL 299 cells. In 22/*n*199 infected cells, the IIi subunit is not efficiently formed. Although 22/*n*199 infected Vero cells do not induce large amounts of IIi, some comigrating immunoreactive material is detectable (Fig. 4.5, lanes 6 and 7). Furthermore, the depletion of the IIo subunit still occurs in Vero (Fig. 4.5, lanes 6 and 7) and HEL 299 cells (Fig. 4.7, lane 8) during 22/*n*199 infection. Because 22/*n*199 produces 199 amino acids of the 420 residue ICP22 protein, it was possible that the 199 peptide was partially functional in RNAP II modifications. The truncated ICP22 protein could be responsible for the depletion of IIo and the remnants of IIi observed in Vero infected 22/*n*199 cells.

To test this hypothesis, an ICP22 null mutant, *d*22, was constructed. Dr. Stephen Rice and Vivian Leong (Lam), University of Alberta, Edmonton, Alberta constructed the *d*22 virus by marker rescue with WT HSV-1 DNA and a plasmid DNA fragment spanning the lac Z gene. Recombinant viruses containing a complete deletion in the ICP22 gene were identified and isolated. HEL 299 cells were infected with *d*22 for 4 and 8 hr. Cell extracts were prepared and then total cell proteins were separated on SDS-PAGE gels. Western blotting was performed with antibodies directed against the large subunit of RNAP II, ARNA3 and 8WG16. HEL 299 cells infected with WT HSV-1 (KOS1.1) showed normal RNAP II modifications, with a loss of IIo and IIa and an induction of IIi. At 4 hr post-infection, *d*22 appeared to induce some IIi (Fig. 4.8, lane 4), but the levels were reduced compared with those observed after KOS1.1 infection at 4 hr (data not shown). At 8 hr post-infection, *d*22 infected HEL 299 cells showed IIa retention, IIo depletion, and little, if any, IIi formation (Fig. 4.8, lane 5 versus lane 3). There was a retention of IIa and a loss of IIo. These results were comparable to those observed in 22/*n*199 infected HEL 299 cells. Because similar results were obtained after 22/*n*199 and *d*22 infection of HEL 299 cells, we conclude that the truncated ICP22 protein produced by 22/*n*199 is not responsible for the small amounts of IIi present at 4 hr post-infection or for the loss of IIo.

The above experiments were repeated with *d*22 infected Vero cells (data not shown). Essentially similar results were obtained. There was no difference in the RNAP II large subunit phosphorylation patterns between *d*22 and 22/*n*199 infected Vero cells. We conclude that the *d*22 and 22/*n*199 mutant infections are similar with respect to RNAP II modifications. The 199 amino acid truncated ICP22 protein is not responsible for the appearance of low amounts of IIi observed early in mutant infection nor for the loss of IIo after mutant infection.

G. Redistribution of RNAP II into viral replication compartments in 22/*n*199 infected cells

The data in Chapter 3 show that RNAP II is recruited into DNA replication compartments after WT HSV-1 infection. In addition, the modification of RNAP II large subunit is not dependent on the relocalization of the enzyme into viral replication compartments, since IIi formation occurs efficiently in cells infected with the ICP8 mutant (Gao and Knipe, 1989). Our results did not indicate whether the converse was true, i.e., that the modification of RNAP II is a prerequisite for its recruitment into DNA replication compartments. We tested whether IIi induction was necessary for localization into viral replication compartments.

Mock, WT HSV-1 (KOS1.1), or 22/*n*199 infected Vero cells were fixed and stained with the RNAP II large subunit antibody, 8WG16, and secondary antibody, GAMR, at 4 and 6 hr post-infection (Fig. 4.9). Consistent with our previous results, RNAP II in mock infected cells was localized in the nucleus in a diffuse, nonnucleolar distribution, with some bright speckles (Fig. 4.9B and Fig. 4.9C). RNAP II in KOS1.1 infected cells was redistributed into DNA replication compartments (Fig. 4.9E and Fig. 4.9F). A similar relocalization of RNAP II occurred in 22/*n*199 infected cells with similar kinetics (Fig. 4.9H and Fig. 4.9I). The subnuclear foci observed in WT HSV-1 and 22/*n*199 infected cells were confirmed to be DNA replication compartments since they costained with 3-83, a polyclonal antibody directed against ICP8, which is a major marker for these compartments (Quinlan et al., 1984) (data not shown).

Because 22/*n*199 growth is more restrictive in other cells lines, we repeated the above immunofluorescence experiments with HeLa S3 and HEL 299 cells (data not shown). Identical results were obtained in HeLa S3 and HEL 299 cells where RNAP II was efficiently localized to viral replication compartments after 22/*n*199 infection with the same kinetics as in Vero cells. We conclude that the recruitment of RNAP II into DNA replication compartments does not require efficient modification of the RNAP II large subunit into the IIi form.

H. Redistribution of RNAP II into viral replication compartments in *d*22 infected cells

Our data show that after 22/*n*199 infection, RNAP II is recruited into DNA replication compartments and that IIi induction is not a prerequisite for localization into these viral compartments. We wanted to ensure that the same phenotype was observed after *d*22 infection. Although IIi formation is not a prerequisite for RNAP II recruitment into DNA replication compartments after infection with 22/*n*199, it is possible that the 199 amino acid ICP22 truncated protein synthesized by 22/*n*199 possesses an activity that enables RNAP II localization into viral compartments. To test this hypothesis, double immunofluorescence experiments were conducted. Vero cells were infected with *d*22 for 5 hr, fixed and stained with primary antibodies directed against the RNAP II large subunit, 8WG16, and ICP8, 3-83, and secondary fluorescence antibodies, GAMR and DARF.

The distribution of RNAP II large subunit after *d*22 infection (Fig. 4.10B) was consistent with our previous findings with 22/*n*199 infection (Fig. 4.9H and Fig. 4.9I). RNAP II was localized into subnuclear foci, which were identified as DNA replication compartments by costaining with ICP8 (Fig. 4.10C). From these results, we conclude that RNAP II localization is similar in *d*22 and 22/*n*199 infections; the ICP22 truncated protein produced in 22/*n*199 infections is not responsible for RNAP II recruitment into DNA replication compartments.

I. Deficiencies in viral gene transcription in 22/*n*199 infected cells

ICP22 mutants exhibit defects in viral gene expression at the level of protein synthesis (Astor et al., Unpublished data; Sears et al., 1985; Poffenberger et al., 1993), particularly in restrictive infections, but some defects in protein expression have also been observed in permissive infections in Vero cells. There is a delay in the synthesis of DE proteins and a reduction in the synthesis of L proteins in ICP22 mutants. For one gene, US11, levels of both mRNA and protein are deficient after ICP22 mutant infections (Purves et al., 1993). Defects in DE and L gene expression exhibited by ICP22 mutants appear to arise from underlying defects in viral gene transcription. Viral gene transcription in Vero cells is relatively efficient in 22/*n*199 infections. However, in more restrictive infections in HEL 299 cells, viral gene (IE, DE and especially L) transcription is reduced compared with that during WT infections. The cell type dependencies for ICP22 during viral transcription suggests that a host cell factor may complement ICP22 function in permissive cells. In addition, defects in viral gene transcription may underly, at least in part, the cell type dependent growth defects observed with ICP22 mutants (Rice et al., 1995).

Photoaffinity labeling experiments demonstrate that the form of RNAP II containing the IIi subunit is active in transcription elongation after WT HSV-1 infection. During 22/*n*199 infections in HEL 299 cells, when IIi induction is inefficient, the transcriptionally active form of RNAP II is a hyperphosphorylated form (Spencer et al., 1997). Although discrete hyperphosphorylated forms are not visible on Western blots of ICP22 mutant infected HEL 299 whole cell extracts (Fig. 4.7, lane 8 and Fig. 4.8, lane 5), a fraction of the hyperphosphorylated forms that remain after ICP22 mutant infection appear to be involved in transcription elongation. The hyperphosphorylated forms observed in photoaffinity labeling experiments must be minor components of total RNAP II, based upon their abundance in Western blot analyses (Spencer et al., 1997)

Expression of HSV-1 L ($\gamma 2$) genes is stimulated by viral DNA replication. This is most dramatically demonstrated by true L genes such as gC and UL36, which are not expressed at detectable levels in the absence of DNA synthesis (Homa et al., 1986; McNabb and Courtney, 1992). It is possible that altered transcription patterns observed in 22/*n*199 infected HEL 299 cells (Rice et al., 1995) are not due to direct effects on transcription but rather from a lower rate of DNA replication. A comparison of the rates at which 22/*n*199 and WT HSV-1 replicated their DNA in infected HEL 299 cells was performed. The 22/*n*199 virus replicates its DNA in HEL 299 cells at a rate that is at least equal to that of WT HSV-1. These experiments show that alterations in transcription observed in 22/*n*199 infected HEL 299 cells can not be explained by differences in viral DNA replication but likely reflect direct effects of ICP22 on viral gene expression (Rice et al., 1995).

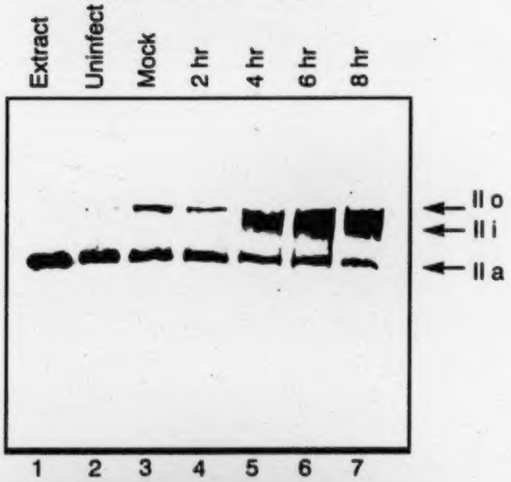
J. Summary

Virus-induced modifications to the phosphorylation state of the RNAP II large subunit (loss of IIo and appearance of IIi) do not require viral DNA replication, recruitment of the enzyme into viral replication compartments, or expression of DE or L genes. In addition, RNAP II modifications do not occur in the presence of virion components alone. The expression of IE proteins, and not just their transcription, appears to be essential in bringing about RNAP II phosphorylation changes.

The IE protein ICP22 is essential for efficient production of IIi. In addition, ICP22 is required for efficient viral DE and L transcription in some cell types. Two or more IE proteins may be redundant in their ability to trigger loss of IIo. In summary, these data suggest that virus-induced modification to the phosphorylation state of RNAP II are multifaceted and involve the actions of several IE proteins.

Figure 4.1. RNAP II localization and modification in Vero cells infected with the HSV-1 ICP8 mutant virus, *d*101. (A) Western blot analysis of the large subunit of RNAP II. Vero cells were uninfected (lane 2), mock infected (lane 3) or infected with the HSV-1 ICP8 mutant virus, *d*101 (lanes 4 to 7), for the times indicated. Cells were harvested and lysed into Laemmli buffer. Extracts were run on SDS-PAGE (6% acrylamide) gels and then immunoblotted. The samples were probed with a mixture of both RNAP II large subunit antibodies, ARNA3 and 8WG16. Lane 1 contained an aliquot of a nuclear *in vitro* transcription extract prepared from HeLa cells. (B) Immunofluorescence analysis of RNAP II distribution. Vero cells were infected with the *d*101 mutant for 5 hr. The cells were fixed and reacted with 8WG16. Secondary antibody was rhodamine-conjugated goat anti-mouse immunoglobulin. Cells were examined under phase-contrast (top panel) or fluorescence microscopy as indicated (bottom panel). Bar = 10 μm.

A. Western - ICP8 mutant


Extract
Uninfect
Mock
2 hr
4 hr
6 hr
8 hr
II o
II i
II a
1
2
3
4
5
6
7

B. Immunofluorescence

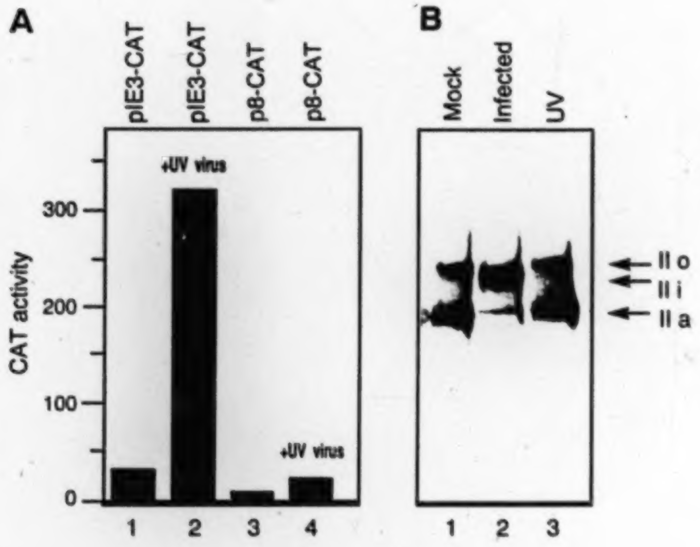


Figure 4.2. UV treatment of HSV-1 virions prevents RNAP II modifications. (A) Transcriptional activation of an IE promoter by UV-inactivated virus stock. Vero cells were transiently transfected with the plasmid pIE3-CAT, which contains the bacterial CAT gene under control of the IE ICP4 gene promoter (lanes 1 and 2), or p8-CAT, which contains the CAT gene under control of the DE ICP8 gene promoter (lanes 3 and 4). Cells were then mock infected or infected with UV-inactivated WT HSV-1 (KOS1.1), as indicated. Extracts were prepared after 24 hr, and the CAT activities were determined. These experiments were performed by Vivian Leong (Lam) and Dr. Stephen Rice, University of Alberta, Edmonton, Alberta. (B) Western blot analysis of RNAP II large subunit. Vero cells were mock infected (lane 1), infected for 5 hr with a mock-UV-irradiated HSV-1 KOS1.1 stock (lane 2), or infected for 5 hr with a UV-inactivated HSV-1 KOS1.1 stock (lane 3). The UV-inactivated virus stock was treated with UV light to crosslink DNA and prevent viral gene expression, as described in Chapters 2 and 4. Extracts were prepared and Western blot analysis was performed as described in the legend of Fig. 4.1. The blot was probed with a mixture of RNAP II large subunit antibodies, 8WG16 and ARNA3.

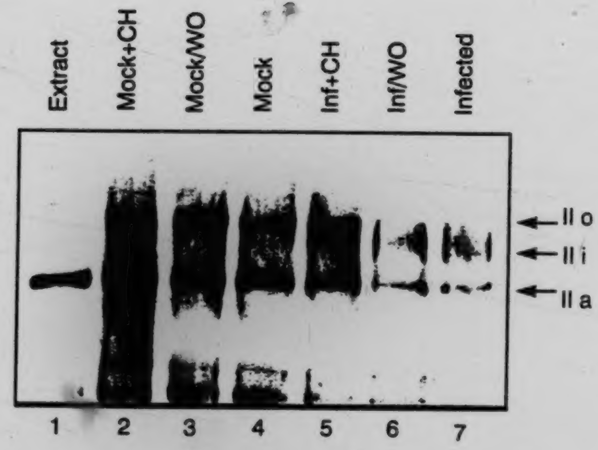


Figure 4.3. RNAP II modification requires viral gene expression. As controls, Vero cells were either mock infected (lane 4) or infected with WT HSV-1 (KOS1.1) for 6 hr in the absence of cycloheximide (lane 7). The remaining cultures were pretreated with cycloheximide for 1 hr before either mock infection in the presence of cycloheximide (lanes 2 and 3) or infection with KOS1.1 in the presence of cycloheximide (lanes 5 and 6). After 6 hr in the continuous presence of cycloheximide, Laemmli extracts were prepared (lanes 2 and 5) or the cells were washed and incubated in drug-free medium for 2 hr (WO) before extracts were prepared (lanes 3 and 6). Cycloheximide was used at a concentration of 50 µg/ml. Western blotting was performed as described in the legend to Fig. 4.1. using the RNAP II large subunit antibody ARNA3. Lane 1 contained an aliquot of a nuclear *in vitro* transcription extract prepared from HeLa cells.

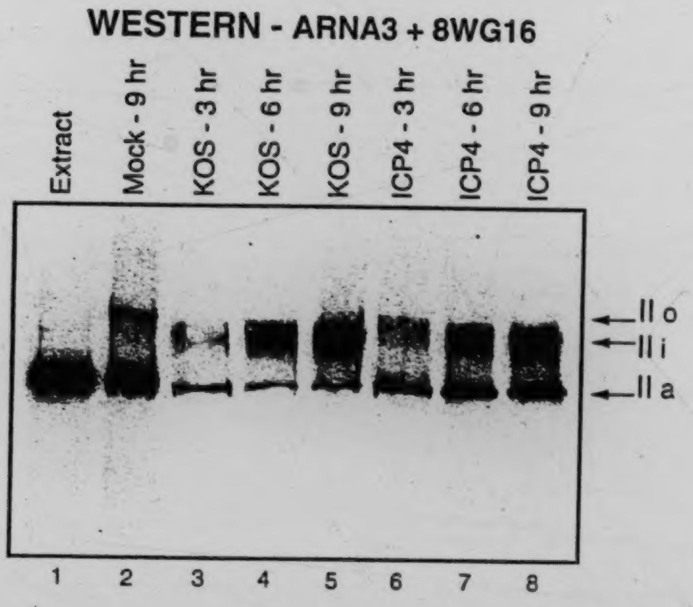
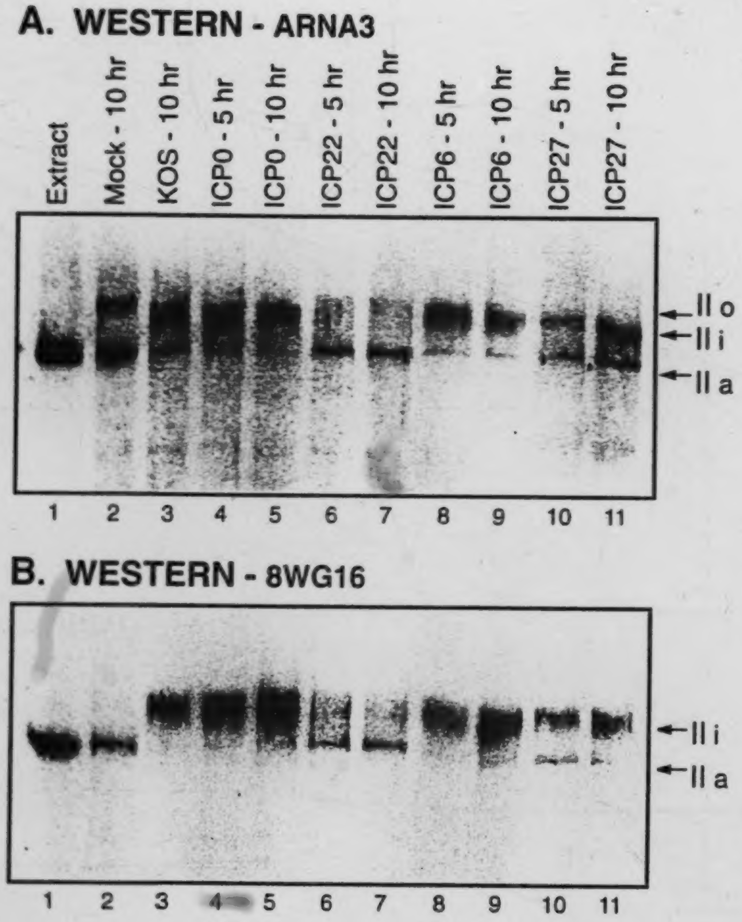

WESTERN - ARNA3 + 8WG16
Extract
Mock - 9 hr
KOS - 3 hr
KOS - 6 hr
KOS - 9 hr
ICP4 - 3 hr
ICP4 - 6 hr
ICP4 - 9 hr
II o
II i
II a
1
2
3
4
5
6
7
8

Figure 4.4. Western blot analysis of the RNAP II large subunit in HSV-1 ICP4 deletion mutant *d*120 infected Vero cells. Vero cells were mock infected (lane 2) or infected with WT HSV-1 (KOS1.1) (lanes 3 to 5) or the HSV-1 ICP4 deletion mutant *d*120 (lanes 6 to 8). Cells were harvested at the times indicated and lysed into Laemmli buffer. Extracts representing equal numbers of cells were run on SDS-PAGE (6% polyacrylamide) gels and analyzed by Western blotting. The blot was probed with a mixture of the RNAP II large subunit antibodies, ARNA3 and 8WG16. Lane 1 contained an aliquot of a nuclear *in vitro* transcription extract prepared from HeLa cells.

Figure 4.5. Western blot analysis of the RNAP II large subunit in Vero cells infected with HSV-1 IE deletion mutants. Vero cells were mock infected (lanes 2) or infected with WT HSV-1 (KOS1.1) (lanes 3), the ICP0 mutant *n*212 (lanes 4 and 5), the ICP22 mutant 22/*n*199 (lanes 6 and 7), the ICP6 mutant ICP6 (lanes 8 and 9), or the ICP27 mutant, *d*27-1 (lanes 10 and 11) for the times indicated. Extracts were prepared and analyzed as described in the legend of Fig. 4.1. Lane 1 contained an aliquot of a nuclear *in vitro* transcription extract prepared from HeLa cells. Blot A was probed with the monoclonal antibody ARNA3, which reacts with the body of the large subunit of RNAP II, whereas blot B was probed with the monoclonal antibody 8WG16, which reacts with the CTD of the large subunit of RNAP II. ARNA3 recognizes all forms of the RNAP II large subunit. 8WG16 recognizes only the IIa and IIi forms.


A. WESTERN - ARNA3
Extract
Mock - 10 hr
KOS - 10 hr
ICP0 - 5 hr
ICP0 - 10 hr
ICP22 - 5 hr
ICP22 - 10 hr
ICP6 - 5 hr
ICP6 - 10 hr
ICP27 - 5 hr
ICP27 - 10 hr
II o
II i
II a
1 2 3 4 5 6 7 8 9 10 11
B. WESTERN - 8WG16
II i
II a
1 2 3 4 5 6 7 8 9 10 11

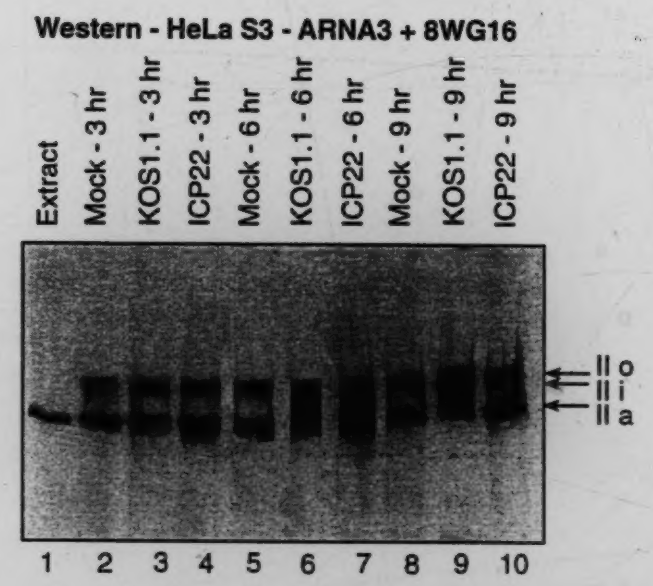

Western - HeLa S3 - ARNA3 + 8WG16
Extract
Mock - 3 hr
KOS1.1 - 3 hr
ICP22 - 3 hr
Mock - 6 hr
KOS1.1 - 6 hr
ICP22 - 6 hr
Mock - 9 hr
KOS1.1 - 9 hr
ICP22 - 9 hr
II o
II i
II a
1
2
3
4
5
6
7
8
9
10

Figure 4.6. Western blot analysis of the RNAP II large subunit in HeLa S3 cells infected with the HSV-1 ICP22 mutant 22/n199. HeLa S3 cells were mock infected (lanes 2, 5, and 8) or infected with W T HSV-1 (KOS1.1) (lanes 3, 6, and 9) or the ICP22 mutant 22/*n*199 (lanes 4, 7, and 10) for the times indicated. Extracts were prepared and analyzed as described in the legend to Fig. 4.1. Lane 1 contained an aliquot of a nuclear *in vitro* transcription extract prepared from HeLa cells. The blots were probed with a mixture of the RNAP II large subunit antibodies, ARNA3 and 8WG16.

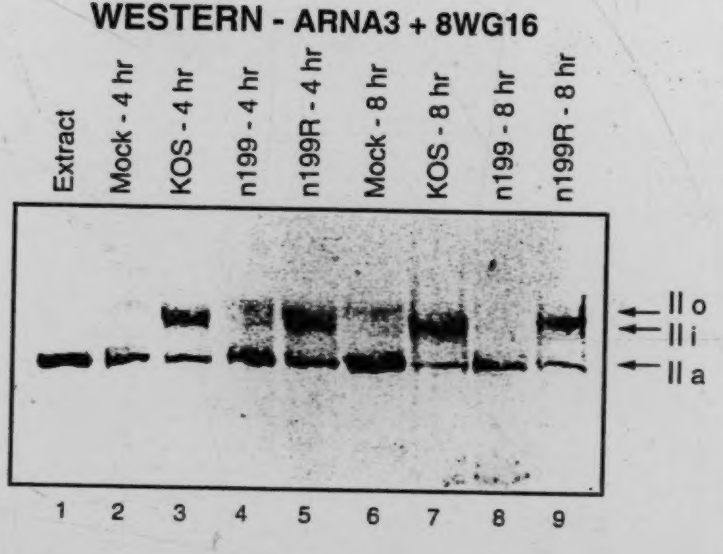

WESTERN - ARNA3 + 8WG16
Extract
Mock - 4 hr
KOS - 4 hr
n199 - 4 hr
n199R - 4 hr
Mock - 8 hr
KOS - 8 hr
n199 - 8 hr
n199R - 8 hr
II o
II i
II a
1
2
3
4
5
6
7
8
9

Figure 4.7. Western blot analysis of the RNAP II large subunit in HEL 299 cells infected with the HSV-1 ICP22 mutant 22/*n*199 and a marker-rescued derivative. HEL 299 cells were mock infected (lanes 2 and 6) or infected with WT HSV-1 (KOS1.1) (lanes 3 and 7), the ICP22 mutant 22/*n*199 (lanes 4 and 8), or a marker-rescued derivative, 22/*n*199R (lanes 5 and 9) for the times indicated. Extracts were prepared and blots were run as described in Fig. 4.1. Lane 1 contained an aliquot of a nuclear *in vitro* transcription extract prepared from HeLa cells. The blot was probed with a mixture of RNAP II large subunit antibodies, ARNA3 and 8WG16.

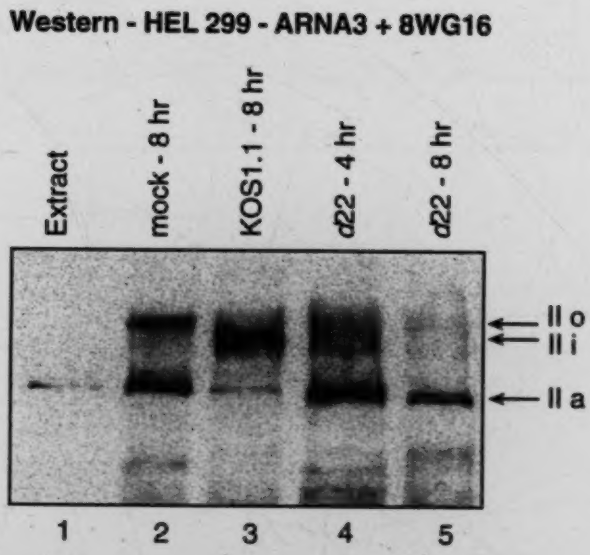
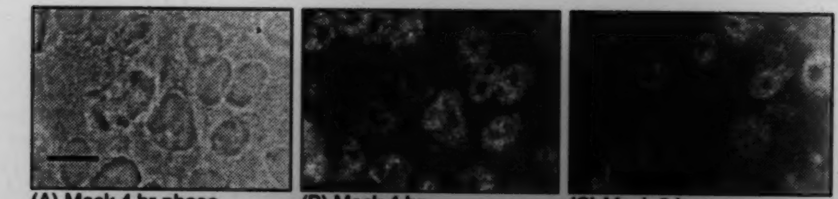
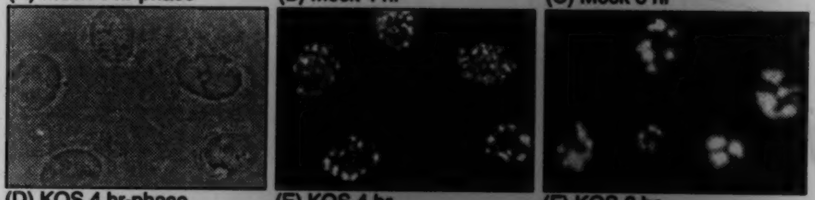

Western - HEL 299 - ARNA3 + 8WG16
Extract
mock - 8 hr
KOS1.1 - 8 hr
*d*22 - 4 hr
*d*22 - 8 hr
II o
II i
II a
1
2
3
4
5

Figure 4.8. Western blot analysis of the RNAP II large subunit in HEL 299 cells infected with the HSV-1 ICP22 mutant *d*22. HEL 299 cells were mock infected (lane 2) or infected with WT HSV-1 (KOS1.1) (lane 3) or the ICP22 mutant *d*22 (lanes 4 and 5) for the times indicated. Extracts were prepared and analyzed as described in Fig. 4.1. Lane 1 contained an aliquot of a HeLa nuclear *in vitro* transcription extract. The blot was probed with a mixture of RNAP II large subunit antibodies, ARNA3 and 8WG16.

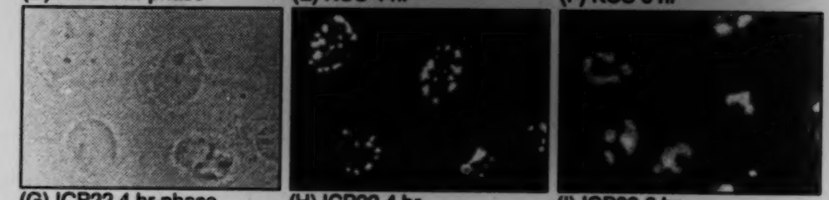
Figure 4.9. Nuclear localization of RNAP II in Vero infected cells. Vero cells were mock infected (A to C) or infected with WT HSV-1 (KOS1.1) (D to F) or 22/*n*199 (G to I). The cells were fixed at the times indicated and processed for immunofluorescence with the primary antibody 8WG16, which recognizes the RNAP II large subunit, and secondary antibody rhodamine-conjugated goat anti-mouse immunoglobulin. Panels A, D, and G are phase-contrast images corresponding to panels B, E, and H respectively. Bar = 10 µm.



(A) Mock 4 hr-phase (B) Mock 4 hr (C) Mock 6 hr



(D) KOS 4 hr-phase (E) KOS 4 hr (F) KOS 6 hr

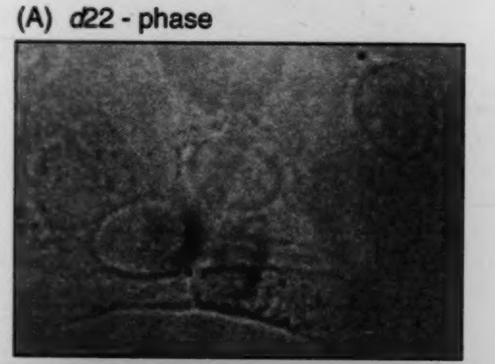


(G) ICP22 4 hr-phase (H) ICP22 4 hr (I) ICP22 6 hr

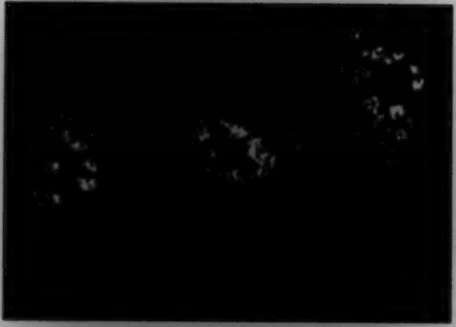
Figure 4.10. Double immunofluorescence of RNAP II and ICP8 in HSV-1 ICP22 mutant *d*22 infected Vero cells. Vero cells were infected with HSV-1 ICP22 mutant *d*22 for 5 hr. The cells were fixed and stained with monoclonal antibody 8WG16 and anti-ICP8 polyclonal antiserum 3-83. Secondary antibodies were a mixture of rhodamine-conjugated goat anti-mouse immunoglobulin (for RNAP II visualization) and fluorescein conjugated donkey anti-rabbit immunoglobulin (for ICP8 visualization). Phase-contrast microscopy (A) of corresponding panels. Detection of RNAP II (B) Detection of ICP8 (C).

Double Immunfluorescence

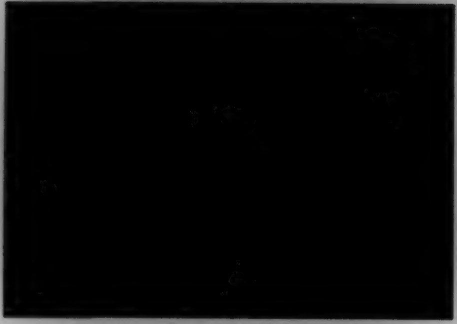
(A) *d22* - phase



(B) *d22* - 8WG16



(C) *d22* - 3-83



CHAPTER FIVE. MODIFICATIONS TO THE RNAP II LARGE SUBUNIT REQUIRE THE PRESENCE OF THE VIRAL KINASE, UL13

A. Introduction

During lytic infection, viral transcription is induced to high levels, while cellular transcription is suppressed (Smiley et al., 1991; Spencer et al., 1997). The transcription of viral genes is dependent on the host's RNAP II transcription machinery. The shift in transcription from cellular to viral genes may involve sequence independent mechanisms such as modifications to RNAP II transcription machinery, alterations in DNA conformation, and localization of viral genes into specialized nuclear compartments (Smiley et al., 1991).

The data described in Chapters 3 and 4 demonstrate that after WT HSV-1 infection, there is a rapid and aberrant phosphorylation of the large subunit of RNAP II, which is dependent on the presence of IE protein ICP22. The mechanism by which ICP22 induces RNAP II modifications is not known. ICP22 may induce IIi formation by altering the activity of an existing cellular CTD kinase or phosphatase. Alternatively, ICP22 could induce the activity of a novel viral or cellular enzyme. In this chapter, I examine the role that viral kinases play in RNAP II modifications.

HSV-1 encodes three protein kinases. These are the products of the ICP6, US3, and UL13 genes (Purves et al., 1986b; Purves et al., 1987; Chung et al., 1989; Conner et al., 1992; Cunningham et al., 1992; Coulter et al., 1993). ICP6 is the large subunit of HSV-1's ribonucleotide reductase whose amino terminal domain has autophosphorylation and serine and threonine kinase activities (Chung et al., 1989; Conner et al., 1992). We originally thought that ICP6 was a promising candidate for the production of IIi, as it is a viral gene product that is expressed early after infection and is not dependent on the presence of ICP4 for its expression (DeLuca et al., 1985; Desai et al., 1993). However, results presented in Chapter 4 demonstrate that ICP6 is not required for RNAP II$_{\mathrm{I}}$ formation, since infection of cells with an ICP6 gene deletion mutant results in WT levels of the IIi subunit.

Less is known about the function of US3. US3 is conserved only among the α-herpesviruses (Baer et al., 1984; McGeoch and Davison, 1986) and is a virion-associated cytoplasmic protein (Frame et al., 1987; Zhang et al., 1990). US3 is involved in the phosphorylation of another virion protein encoded by the UL34 gene (Purves et al., 1991; Purves and Roizman, 1992). There is some evidence that ICP22 phosphorylation is dependent on US3 (Purves et al., 1993). In support of this, ICP22 possesses three potential target sites for phosphorylation by the US3 kinase (McGeoch et al., 1985; Purves et al., 1986b).

UL13 is a 55 kDa virion-associated protein containing serine and threonine kinase activity (Smith and Smith, 1989; Cunningham et al., 1992; Overton et al., 1992). It has homologues in the α-, β- and γ-herpesviruses (McGeoch, 1989). UL13 is a (β) "late" gene product whose expression commences between 3 and 6 hr post-infection and continues beyond 9 hr post-infection. Although categorized as a β gene product, the synthesis of UL13 mRNA shows a similar pattern to that observed for the L gene ICP5 (Costa et al., 1983; Overton et al., 1992). UL13 possesses autophosphorylation abilities (Overton et al., 1992) in addition to phosphorylating the tegument protein, VP22 (Coulter et al., 1993). Past work from Roizman's group established a link between ICP22 phosphorylation and the presence of UL13 (Purves and Roizman, 1992). HSV-1 mutants with a deletion in the UL13 gene do not show the normal pattern of ICP22 phosphorylation, demonstrating that the phosphorylation of ICP22 is dependent on UL13 (Purves and Roizman, 1992). This suggests that ICP22 is itself a substrate for the UL13 protein kinase. However, it has not been firmly demonstrated whether ICP22 phosphorylation by UL13 is direct or is mediated by other cellular or viral proteins. It is believed that the phosphorylation of ICP22 by UL13 is distinct from the phosphorylation of ICP22 by US3 (Purves et al., 1993). In addition, UL13 mutant viruses display similar cell-type dependencies for growth to those of ICP22 mutant viruses (Purves et al., 1993).

UL13 mutant infected cells display reduced levels of certain viral proteins, especially L proteins (Purves et al., 1993). Expression of the IE gene ICP0 and the L gene US11 has been examined at the RNA level (Purves et al., 1993) by Northern blotting. The mRNA for these two genes remains low after UL13 mutant infection in baby hamster kidney cells. However, because Northern blotting measures only the levels of steady-state RNA, as opposed to specific transcription, it is not possible to determine the effects of UL13 on transcription in these studies.

As described in Chapter 4, DE and L proteins are not required for IIi induction. In an ICP4 mutant infection, where DE and L gene expression is absent, RNAP II modifications occur efficiently. If UL13, being a β "late" protein (Costa et al., 1983; Overton et al., 1992), behaves as other DE and L proteins and is transcriptionally stimulated by ICP4, then the presence of UL13 protein produced late in infection would not be expected to be required for IIi induction.

In Chapter 4, we also demonstrate that virion components alone are not sufficient to cause RNAP II modifications and that viral IE protein (i.e. ICP22) synthesis is required for the induction of the IIi form. In addition to being produced late in infection, UL13 enters the cell as a component of the virion (Purves et al., 1986a; Frame et al., 1987; Zhang et al., 1990). Since UL13 protein kinase is virion associated, we expect that its presence alone during the onset of infection is not sufficient to induce IIi. However, UL13 may work in combination with ICP22 to modify RNAP II large subunit. Based on what is known about ICP22 (its phosphorylation by UL13 and its role in RNAP II modifications), we hypothesize that UL13 also plays a role in the induction of IIi. Our hypothesis is that IIi formation would be deficient in cells infected with a UL13 mutant. UL13 may indirectly induce IIi by causing ICP22 phosphorylation, which in turn may be essential for RNAP II modifications. This scenario is possible because ICP22 phosphorylation is detected as early as 4 hr post-infection (Purves et al., 1993), which is coincident with IIi formation. In addition to this, UL13 may function as an ICP22 dependent CTD kinase.

In the experiments reported in this chapter, I address the questions of whether UL13 is required for normal viral transcription patterns, for repression of cellular transcription, and for modifications to RNAP II.

B. IIi induction requires the viral kinase, UL13

To determine whether UL13 is required for the induction of IIi, we analyzed RNAP II modifications in HEL 299 cells infected with an HSV-1 UL13 deletion mutant, *d*UL13, which encodes sequences for only the first

154 amino acids of the 518 amino acid UL13 protein. The *d*UL13 virus was constructed by Dr. Stephen Rice, University of Alberta, Edmonton, Alberta, by marker transfer with WT HSV-1 DNA and the pBgl OZ-alpha plasmid, which contains the lac Z gene with flanking N terminal UL13 protein-coding sequences and non-protein-coding 3′ UL13 sequences. Cell extracts were harvested at 4 and 8 hr post-infection, and total proteins were separated on SDS-PAGE gels and analyzed by Western blotting with antibodies directed against the large subunit of RNAP II: ARNA3 and 8WG16 (Fig. 5.1). Infection with WT HSV-1 (KOS1.1) resulted in modifications to RNAP II as seen previously (Fig. 5.1). However, infection with *d*UL13 resulted in inefficient RNAP II modifications, as the levels of IIi were significantly reduced (Fig. 5.1, lanes 4 and 5) compared with those of the KOS1.1 infection (Fig. 5.1, lane 3). Some faint comigrating immunoreactive material similar to IIi was observed at 4 hr post-infection (Fig. 5.1, lane 4), but not at 8 hr post-infection with the UL13 mutant virus (Fig. 5.1, lane 5). After *d*UL13 infection, the IIa form was retained, but the IIo form was severely depleted. These results indicate that UL13, like ICP22, is required for IIi induction in HEL 299 cells.

We confirmed the requirement for both UL13 and ICP22 in RNAP II modifications by performing Western blotting with extracts prepared from cells infected with 22/*n*199, *d*22, or *d*UL13 in one experiment (data not shown). The loss of IIi and IIo and the retention of IIa occurred similarly in 22/*n*199, *d*22, and *d*UL13 infected cells. Therefore, both UL13 protein kinase and ICP22 are necessary for the efficient induction of the IIi subunit in HEL 299 cells.

ICP22 mutants and UL13 mutants have common phenotypic qualities in that they possess similar cell dependent replication defects (Astor et al., Unpublished data; Sears et al., 1985; Poffenberger et al., 1993; Purves et al., 1993). ICP22 mutants grow efficiently in some cell lines, such as Vero cells, but not in others, such as baby hamster kidney cells or HEL cells (Astor et al., Unpublished data; Sears et al., 1985; Poffenberger et al., 1993). Likewise, UL13 mutants display similar behavior in that they grow efficiently in human epidermoid carcinoma and Vero cells but not in baby hamster kidney cells and HEL cells (Purves et al., 1993).

We next analyzed RNAP II modifications in *d*UL13 infected Vero cells, which are permissive for UL13 mutant growth (Purves et al., 1993) (data not shown). We found that infection of Vero cells with *d*UL13 resulted in inefficient IIi induction, similar to that observed in Vero cells infected with 22/*n*199 (Fig. 4.5A and 4.5B, lanes 6 and 7) or *d*22 (data not shown). There was a loss in IIo and IIi and a retention of IIa. The *d*UL13 virus appeared to induce low but detectable levels of IIi at late times (data not shown) as observed in 22/*n*199 (Fig. 4.5, lane 7) and *d*22 (data not shown) infected Vero cells. Therefore, UL13 and ICP22 are both required for efficient RNAP IIi induction, not only in HEL 299 cells but also in Vero cells.

Although infection with ICP22 and UL13 mutant viruses does not result in efficient IIi production, the IIo form of the large subunit is depleted. As described in Chapter 4, there is a loss of the IIo form after infection of cells with viruses mutant in each of the IE genes. Therefore, it is possible that more than one IE protein may be redundant in their ability to cause IIo depletion.

C. Redistribution of RNAP II into viral replication compartments in *d*UL13 infected cells

Results in Chapters 3 and 4 demonstrate that RNAP II is recruited into DNA replication compartments after WT HSV-1 and ICP22 mutant virus infection. In order to determine whether UL13 is required for recruitment of RNAP II into viral replication compartments, double immunofluorescence experiments were conducted. Vero cells were infected with *d*UL13 for 5 hr, fixed and stained with primary antibodies directed against the RNAP II large subunit (8WG16) and ICP8 (3-83) and secondary fluorescence antibodies, GAMR and DARF. RNAP II was localized into subnuclear foci after *d*UL13 infection (Fig. 5.2B), as observed after 22/*n*199 (Fig. 4.9H and Fig. 4.9I) and *d*22 (Fig. 4.10B) infection. These nuclear foci were identified as DNA replication compartments by costaining with ICP8 (Fig. 5.2C). From these results, we conclude that the recruitment of RNAP II into viral replication compartments does not require UL13. Furthermore, these results further confirm that the efficient modification of the RNAP II large subunit into the IIi form is not required for the localization of RNAP II into DNA replication compartments.

D. Viral gene transcription in *d*UL13 infected cells

We demonstrated that 22/*n*199 displays defects in viral gene expression in certain cell lines (Rice et al., 1995). Viral gene transcription is relatively efficient after 22/*n*199 infection of Vero cells. In a more restrictive infection, such as that in HEL 299 cells, viral gene transcription is impaired after 22/*n*199 infection (Rice et al., 1995). Because UL13 and ICP22 mutants display similar phenotypes in terms of cell type dependent growth (Purves et al., 1993), inefficient IIi induction (Chapter 4 and this chapter), and RNAP II relocalization after infection (Chapter 4 and this chapter), I was interested in examining viral transcription in *d*UL13 infected cells. I sought to determine if UL13 and ICP22 mutants show commonalties in terms of viral transcription in specific cell lines.

To measure transcription rates of a variety of viral genes after *d*UL13 infection, I used the nuclear run-on transcription assay. To date, the nuclear run-on transcription assay is the only method developed to directly measure RNAP activity on specific genes. The nuclear run-on transcription assay does not specifically measure initiation but rather measures the location of a transcriptionally engaged RNAP II ternary (RNAP II, DNA, and RNA) complex that is elongating or paused but released during the assay (Leys et al., 1984).

Other transcription assays such as whole cell pulse-labeling *in vivo* measure the effects of both RNA synthesis and rapid degradation (Spencer et al., 1997). The nuclear run-on transcription assay is free from mRNA degradation and processing events, because isolated nuclei are utilized. Cellular nucleases are removed, thereby limiting RNA degradation. In addition, the experimental conditions used in nuclear run-on transcription assays are not favorable for RNA processing (Leys et al., 1984; Schilling and Farnham, 1994).

Viral gene transcription was examined in Vero cell nuclei isolated from WT HSV-1 (KOS1.1) or *d*UL13 infected cells at 3, 6, and 9 hr post-infection. RNA transcripts initiated *in vivo* were elongated *in vitro* in the presence of ^{32}P-UTP, and the radiolabeled run-on transcripts were hybridized to single-stranded DNAs complementary to either specific viral mRNAs (sense (S) probes) or antisense RNAs from the same regions (antisense (AS) probes). The probes used detected transcription of two IE genes (ICP4 and ICP27), two DE genes (ICP8 and polymerase [pol]), and three L genes (VP16, gC, and UL36). The buffer utilized during the transcription assay contained 150 mM potassium chloride, which is adequate to release paused RNAPs (Rougvie and Lis, 1988; Krumm et al., 1992). Therefore, the run-on signals in the transcription assays represented both elongating and paused (but released during the assay) RNAP II.

The WT HSV-1 (KOS1.1) transcription pattern (Fig. 5.3A) was largely consistent with the results of previous nuclear run-on analyses (Godowski and Knipe, 1986; Weinheimer and McKnight, 1987; Rice et al., 1995). At 3 hr post-infection, specific sense transcription occurred and by 6 hr post-infection, there were high levels of IE, DE, and L sense transcription. Apparent antisense transcription occurred in all regions of the viral genome by 6 and 9 hr post-infection The phenomenon of antisense transcription of the HSV-1 genome has been observed in previous nuclear run-on studies and appears to be contingent upon active viral DNA replication (Godowski and Knipe, 1986; Weinheimer and McKnight, 1987; Rice et al., 1995). The transcription pattern in *d*UL13 infected Vero cells (Fig. 5.3B) appeared generally similar to that in KOS1.1 infected cells. At 3 hr post-infection, a WT pattern of sense transcription was observed, although overall levels were lower with all probes. At 6 hr, levels of ICP27, ICP8, and polymerase sense transcription were lower than in a WT infection. Antisense levels were lower on all probes except on polymerase. Our results do not agree with previous experiments which indicate that polymerase antisense transcription is lower than sense transcription (Weinheimer and McKnight, 1987). At 9 hr post-infection, transcription patterns on all probes appeared similar in WT and *d*UL13 infected cells. To quantitate the effects of UL13 mutation on transcription in Vero cells, the signals from the nuclear run-on experiment in Fig. 5.3 were analyzed by PhosphorImage, focusing on the ICP4 (Fig. 5.4A), ICP8 (Fig. 5.4B), and gC (Fig. 5.4C) genes as representative members of the IE, DE and L gene classes respectively. At 3 hr post-infection, there were low levels of ICP4 sense transcription in KOS1.1 infected Vero cells (Fig. 5.4A). Previous nuclear run-on transcription assays demonstrate that ICP4 sense transcription fluctuates in KOS1.1 infected Vero cells. High levels of ICP4 sense transcription are observed at 2 hr post-infection. ICP4 sense transcription decreases from 2 to 4 hr post-infection and increases from 4 to 6 hr post-infection in KOS1.1 infected Vero cells (Godowski and Knipe, 1986). It is likely that ICP4 transcription has peaked and declined by 3 hr post-infection. There did not appear to be any major impairment in L viral transcription (sense and antisense) in *d*UL13 infected Vero cells at 9 hr (all data not shown). L sense transcription levels at 6 and 9 hr post-infection were very similar in KOS1.1 and *d*UL13 infected cells. The antisense transcription of gC varied slightly at 6 hr and 9 hr post-infection between KOS1.1 and *d*UL13. In *d*UL13 infected Vero cells, ICP4 and ICP8 sense transcription is lower than in KOS1.1 infected Vero cells at 6 hr. This is especially true for ICP4 sense transcription, which is as low as ICP4 antisense transcription at 3 and 6 hr post-infection. However, by 9 hr post-infection levels of ICP4 and ICP8 sense transcription in *d*UL13 mutant infected Vero cells is comparable to KOS1.1 infected Vero cells. There appears to be a delay in IE and DE sense transcription that reaches WT levels by 9 hr post-infection in a *d*UL13 mutant infection. Antisense transcription for ICP4 and ICP8 is similar in KOS1.1 and dUL13 infected cells. These results indicate that UL13 is not required for normal viral transcription (sense and antisense) in Vero cells by 9 hr post-infection. Because IIi is not efficiently induced in a *d*UL13 infection, it appears that the presence of IIi is not required for viral gene transcription in Vero cells.

Previous experiments have shown that ICP22 and UL13 mutant viruses grow efficiently in Vero cells (Purves et al., 1993). The data here show that viral transcription (sense and antisense) in Vero cells after *d*UL13 infection is similar to that in Vero cells after 22/*n*199 infection (Rice et al., 1995).

I next examined *d*UL13 transcription (sense and antisense) patterns in another cell line. Nuclear run-on assays were performed on nuclei isolated from HeLa S3 cells infected with WT HSV-1 (KOS1.1) or *d*UL13 for 3, 6 and 9 hr. The WT HSV-1 transcription patterns (Fig. 5.5A) were similar to those observed in Vero cells (Fig. 5.3A). At 3 hr post-infection, sense transcription occurred in IE, DE, and L genes. By 6 hr post-infection, there was abundant IE, DE, and L gene expression in addition to high levels of antisense transcription in all regions of the genome. The transcription pattern in *d*UL13 infected HeLa S3 cells (Fig. 5.5B) was different from the WT HSV-1 transcription pattern (Fig. 5.5A) in two major respects. Firstly, sense transcription in all gene classes, especially in all three L genes, gC, VP16, and UL36, was reduced at 6 and 9 hr post-infection. IE and DE gene transcription also appeared to be reduced but to a lesser extent. Secondly, there was less antisense transcription after *d*UL13 infection at both 6 and 9 hr post-infection. To quantitate the effects of the UL13 mutation on transcription in HeLa S3 cells, the signals from the nuclear run-on experiments in Fig. 5.5 were analyzed by a PhosphorImager, focusing on the ICP27 (Fig. 5.6A), ICP8 (Fig. 5.6B) and VP16 (Fig. 5.6C) genes as representative members of the IE, DE, and L gene classes respectively. Sense strand transcription was reduced for all three genes examined at all time points. However, the difference was most dramatic for the L VP16 gene (Fig. 5.6C). Sense transcription of the VP16 gene at 6 and 9 hr post-infection in the *d*UL13 infection was reduced between two- and threefold compared with that of the WT HSV-1 infection. At 9 hr, the low level of VP16 gene sense strand transcription in the *d*UL13 infected cells was essentially equal to the levels of VP16 antisense transcription in the KOS1.1 infected cells. In contrast, VP16 gene sense transcription in WT HSV-1 infected cells was three and a half- to sixfold higher than the antisense transcription levels at 6 and 9 hr respectively. In all three genes, the levels of antisense transcription at 6 and 9 hr post-infection were lower (by two- to two and a half-fold) in *d*UL13 infected HeLa S3 cells compared with that of KOS1.1 infected cells. These results indicate that UL13 is required for normal viral transcription (sense and antisense) patterns in HeLa S3 cells. Because IIi is not efficiently induced in a *d*UL13 infection, the presence of IIi may be required for the transcription of viral genes in HeLa S3 cells.

The viral transcription (sense and antisense) patterns after *d*UL13 infection closely resembled viral transcription patterns after 22/*n*199 infection in HeLa S3 cells (Spencer, 1994, Unpublished data). To date, there have been no experiments examining growth efficiencies of ICP22 and UL13 mutants in HeLa S3 cells.

I then set out to examine viral transcription after *d*UL13 infection of HEL 299 cells, which are known to be restrictive for both UL13 and ICP22 mutant virus growth (Purves et al., 1993). In one nuclear run-on experiment, I compared viral transcription in KOS1.1, *d*UL13, and 22/*n*199 infected HEL 299 cells (Fig. 5.7). The WT HSV-1 transcription pattern (Fig. 5.7A) was similar to that observed in Vero (Fig. 5.3A) and HeLa S3 cells (Fig. 5.5A). The transcription patterns of viral genes after infection of HEL 299 cells with *d*UL13 and 22/*n*199 (Fig. 5.7B and 5.7C respectively) were somewhat different from the WT HSV-1 pattern. Sense transcription of all gene classes was reduced at 6 and 9 hr post-infection. In addition, there was less antisense transcription after *d*UL13 and 22/*n*199 infection at both 6 and 9 hr.

To quantitate the transcription in HEL 299 cells infected with UL13 and ICP22 mutant viruses, the signals from the nuclear run-on experiments in Fig. 5.7 were analyzed by PhosphorImager, focusing on the ICP4 (Fig. 5.8A and 5.9A), ICP8 (Fig. 5.8B and 5.9B) and VP16 (Fig. 5.8C and 5.9C) genes as representative members of the IE, DE, and L gene classes respectively. Sense strand transcription was reduced on all three genes examined (Fig. 5.8) at 6 and 9 hr post-infection in both *d*UL13 and 22/*n*199 infected cells. The data from all genes indicated that the levels of antisense transcription at 6 and 9 hr post-infection were lower (by two- to fourteenfold) in *d*UL13 infected cells compared to KOS1.1 infected cells (Fig. 5.9). The levels of antisense transcription at 6 and 9 hr post-infection were also lower (by three to four and a half-fold) in 22/*n*199 infected cells compared to KOS1.1 infected cells. These results indicate that UL13 and ICP22 are required for normal viral transcription (sense and antisense) patterns in HEL 299 cells. Because IIi is not efficiently induced in a *d*UL13 or 22/*n*199 infection, the presence of IIi may be required for normal levels of transcription of viral genes in HEL 299 cells.

Our results show that viral transcription in a *d*UL13 infection is similar to that in a 22/*n*199 infection. The results observed for the 22/*n*199 virus confirm previous data that show lower levels of IE, DE, and especially L gene transcription after a 22/*n*199 infection in HEL 299 cells (Rice et al., 1995).

It is possible that the altered transcription patterns observed in *d*UL13 infected HEL 299 cells (Rice et al., 1995) are not due to direct effects on transcription but rather from a lower rate of DNA replication. The expression of HSV-1 L ($\gamma 2$) genes and antisense transcription are stimulated by viral DNA replication (Godowski and Knipe, 1986; Homa et al., 1986; McNabb and Courtney, 1992). We have not performed experiments to address this issue, although deficiencies in transcription are not the result of impairments in DNA replication in ICP22 mutant infected HEL cells (Rice et al., 1995).

The impairment of *d*UL13 in viral transcription appears to be cell-type specific. In HEL 299 cells where *d*UL13 growth is restrictive, UL13, and perhaps RNAP II modifications, contribute to efficient viral transcription. In Vero cells where *d*UL13 growth is permissive, UL13 and RNAP II modifications may not be required for efficient viral transcription. Host cell factors in Vero cells may complement the ICP22 or UL13 defect. These results suggest that a defect in viral gene transcription may underly, at least in part, the cell type dependent growth defects exhibited by 22/*n*199 and *d*UL13. In *d*UL13 infected HeLa S3 cells, the level of viral transcription impairment is less severe than in *d*UL13 infected HEL 299 cells. We have not examined growth of *d*UL13 in HeLa S3 cells. However, if there is a correlation between efficiency of viral DE and L gene transcription and growth efficiency, I would expect *d*UL13 to grow better in Vero cells than in either HeLa S3 or HEL 299 cells.

Antisense transcription was reduced in *d*UL13 infected HEL 299 and HeLa S3 cells. Antisense transcription refers to transcription of the opposite strand of DNA that produces normal RNA transcripts that encode proteins. Previous studies show that antisense transcription of the HSV-1 genome is dependent on viral DNA replication, since it is not observed at early times after infection or when infection is carried out in the presence of viral DNA synthesis inhibitors (Godowski and Knipe, 1986; Weinheimer and McKnight, 1987). Defects in antisense transcription in 22/*n*199 infected HEL cells do not appear to be due to defects in viral DNA replication, as viral DNA synthesis is efficient in ICP22 mutant infected cells (Rice et al., 1995). In infected HEL 299 cells, antisense transcription may be dependent not only on viral DNA replication, but also on functional ICP22 and UL13. The significance of these findings is unclear, since the biological relevance of antisense transcription is not understood. It is possible that antisense transcription is the result of promiscuous RNAP II molecules lacking transcriptional specificity after viral genome replication (Rice et al., 1995).

There is support for this view in the literature. Zhang and Wagner (1987) studied HSV-1 gene transcription by pulse-labeling techniques, where [^{3}H]uridine incorporated into nuclear RNA was measured. They found that transcription of several IE and DE genes declined at late times in infection, suggesting that nonspecific transcription was not occurring at late times. Antisense RNA was not detected in their experiments, but it is possible that antisense transcription occurs *in vivo*, since significant RNA turnover can occur in pulse-labeling experiments, but RNAs produced during nuclear run-on assays are stable (Leys et al., 1984; Schilling and Farnham, 1994). If antisense transcripts are rapidly degraded in an intact cell, they may not be detected by pulse-labeling.

E. Cellular gene transcription is efficiently repressed in *d*UL13 infected cells

UL13 and ICP22 mutant viruses display similar phenotypes in terms of cell type dependent growth (Purves et al., 1993), inefficient IIi induction (Chapter 4, this chapter), RNAP II localization after infection (Chapter 4, this chapter), and viral transcription in specific cell types (this chapter). I next sought to investigate the role UL13 plays in the repression of cellular transcription after infection.

Lytic infection of cells with WT HSV-1 results in a rapid repression of host gene transcription and the activation of viral gene transcription. This shift in transcription is thought to involve the diversion of the host's RNAP II transcription machinery from the cellular genome to the viral genome (Smibert and Smiley, 1990; Smiley et al., 1991; Spencer et al., 1997). The inhibition of cellular gene transcription requires the expression of IE genes (Smiley et al., 1991; Spencer et al., 1997). Repression of cellular gene transcription is dependent on the expression of multiple IE genes and not ICP22 alone. Cellular RNAP II transcription is as efficiently suppressed in cells infected with the ICP22 mutant virus, 22/*n*199, as in cells infected with

WT HSV-1. ICP22 does not affect the shut-off of cellular gene transcription, even in cells restrictive for growth of the ICP22 mutant. Since ICP22 has been found to be necessary for efficient IIi induction, the modification of RNAP II large subunit to the IIi form appears to be unlinked to virus-induced repression of host transcription (Spencer et al., 1997).

Previous studies claim that UL13 is required for host shut-off of protein synthesis (Overton et al., 1994). Steady-state levels of ^{35}S-labeled actin protein is reduced in cells infected with WT HSV-1 but not in those infected with UL13 mutant viruses. In these experiments, the levels of protein were examined but specific cellular transcription after UL13 mutant infection was not tested.

In order to analyze cellular gene transcription, I performed nuclear run-on transcription assays using nuclei isolated from HeLa S3 cells that were mock infected or infected with WT HSV-1 (KOS1.1) or *d*UL13 (Fig. 5.10). Filters contained single-stranded M13 DNA probes that detect either sense or antisense transcription from several endogenous cellular genes. DNA probes detecting transcription from 5′ and 3′ regions of the c-myc, c-fos, and γ-actin genes were used. The transcription signals from the 5′ ends are distinct from the 3′ ends of these genes. Transcripts elongated during the nuclear run-on transcription assay are typically short, between 240 and 400 bases. The synthesized transcripts are hybridized to DNA probes corresponding to 5′ and 3′ gene regions. The transcripts that have extended into the 3′ region may hybridize to both 5′ and 3′ DNA probes. However, RNase A digestion during the washing procedure ensures that the signal observed corresponds to specific gene regions transcribed during the nuclear run-on transcription assay.

The transcription pattern on these cellular genes in mock infected cells was similar to that observed previously in HeLa cells (Spencer et al., 1997). In mock infected cells (Fig. 5.10A), sense transcription occurred within the c-myc, c-fos, γ-actin, GAPDH and histone H2b genes. The c-myc gene was transcribed in exon 1 (E1) and into the intron 1 (I1) region. This pattern has been observed in other transformed human cell lines and is correlated with the presence of high levels of c-myc steady-state RNA (Spencer et al., 1990). Transcription of the human c-myc gene is regulated partially by controlling the amount of promoter-paused RNAP II that is released into full length transcription (Spencer and Groudine, 1990; Krumm et al., 1995). The c-fos transcription pattern did not display the typical transcription block pattern, where most transcription at the 5′ end of the c-fos gene does not elongate into the exon 4 region (Spencer et al., 1997). In this particular experiment, there was considerable read-through transcription into the c-fos 3′ region (50%). A block to transcription elongation in hamster and murine c-fos genes has been documented and release of this block may be one mechanism controlling levels of steady-state c-fos mRNA (reviewed in Spencer and Groudine, 1990). Increased read-through transcription into the c-fos 3′ region occurs when HeLa cells are stimulated to enter the cell cycle from quiescence (Spencer, 1996, Unpublished data). The γ-actin gene showed typical read-through transcription, indicating that most transcription complexes traversed the gene from the promoter region to beyond exon 4. The GAPDH and histone H2b probes were designed to detect transcription throughout the gene and thus, transcription elongation patterns could not be examined. Antisense transcription was also detected in the c-fos 3′ region. The intensity of these antisense signals have been found to vary from experiment to experiment (Spencer et al., 1997).

In WT HSV-1 infected cells, RNAP II transcription of all cellular genes declined over a 9 hr infection period (Fig. 5.10B). Quantitation of nuclear run-on signals by PhosphorImager analysis indicated that RNAP II transcription on most of these cellular genes declined to less than 9% of mock levels by 9 hr post-infection with KOS1.1 (Table 5.1, Fig. 5.11). An exception was the γ-actin 5′ region, which retained higher levels of transcription (51.2% of mock levels) at 9 hr post-infection. The transcription pattern observed was generally similar to that observed in previous studies in WT HSV-1 infected HeLa S3 cells (Spencer et al., 1997), where efficient cellular transcription repression occurred after infection.

In *d*UL13 infected cells (Fig. 5.10C), transcription signals on all probes declined as infection proceeded, similar to KOS1.1 infected cells (Fig. 5.10B). Quantitation of nuclear run-on signals by PhosphorImage analysis indicated that RNAP II transcription on most cellular genes was reduced to less than 12% of mock levels by 9 hr post-infection with *d*UL13 (Table 5.1, Fig. 5.11). An exception was the γ-actin 5′ region, which retained higher levels of transcription (68.9% of mock levels) at 9 hr post-infection. These results indicate that virus-induced repression of the host cell transcription occurs efficiently in either the absence or presence of UL13.

In order to directly compare cellular transcription rates in *d*UL13 and 22/*n*199 infections, I next examined cellular transcription in *d*UL13 and 22/*n*199 infected HEL 299 cells in the same experiment. HEL 299 cells were mock, WT (HSV-1), KOS1.1, *d*UL13, or 22/*n*199 infected for 3, 6 and 9 hr. Nuclei were harvested and nuclear run-on experiments were performed on equal numbers of nuclei/sample using the same cellular gene probes as before (Fig. 5.12). The patterns of transcription in mock infected HEL 299 cells were similar to those observed in Fig. 5.10A with the exception of the c-myc (E1 and I1) and c-fos 3′ gene probes. In this experiment, the c-fos transcription pattern showed a typical transcription block pattern, where most transcription at the 5′ end of the gene did not elongate into the exon 4 region. This pattern has been observed frequently in HeLa S3 cells and HEL cells (Spencer et al., 1997). The c-myc (E1 and I1) transcription levels are lower in HEL 299 cells as compared to HeLa S3 cells. This has also been observed previously (Spencer et al., 1997). As infection proceeded with KOS1.1 (Fig. 5.12B), *d*UL13 (Fig. 5.12C) and 22/*n*199 (Fig. 5.12D), transcription signals on all cellular probes declined. One exception was the γ-actin 5′ gene probe which showed higher levels of transcription after W T and *d*UL13 virus infection. These results indicate that both UL13 and ICP22 may not be required for the down-regulation of cellular gene transcription.

The signals from the nuclear run-on in Fig. 5.12 were quantitated by PhosphorImage analysis (Table 5.2, Fig. 5.13). RNAP II transcription on most of these cellular genes declined to less than 20% of mock levels after 9 hr infection with all three viruses. Exceptions were the c-myc I1 and c-fos 3′ regions which were close to background levels on all filters and were therefore difficult to evaluate. The other exception was γ-actin 5′ region, which only decreased to 66% of mock levels at 9 hr post-infection in 22/*n*199 and increased to 116.8% and 123.1% of mock levels at 9 hr post-infection in KOS1.1 and *d*UL13 infected cells. Different cellular genes experienced different levels of repression. Despite such differences, the virus-induced shut-off is efficient in ICP22 and UL13 mutant infections to similar degrees as in WT HSV-1 infections. The levels of transcription, as expressed as percent of mock, in KOS1.1, *d*UL13, and 22/*n*199 infected cells, did not differ by more than 10 to 15% in most genes. These results indicate that the degree and efficiency of cellular repression is similar after infection with all three viruses. Our transcription results for 22/*n*199 also confirm previous results examining cellular transcription in 22/*n*199 infected cells (Spencer et al., 1997).

These results differ from those of Kemp and Latchman (1988a) who reported that transcription repression on a number of unidentified cellular cDNA's were dependent on ICP22 expression. In my experiments and also those reported by Spencer et al. (1997), ICP22 had little if any effect on the transcription repression of host genes. It is possible that differences between our studies and those of Kemp and Latchman (1988a) are due to different cell lines and virus strains used.

F. Summary

The virion associated protein kinase UL13 is required for efficient modification to RNAP II_I but is not required for the recruitment of RNAP II into nuclear viral replication compartments. The lack of efficient IIi formation after infection with the UL13 mutant is very similar to that observed in cells infected with ICP22 mutants. IIi induction is dependent not only on ICP22 but also on UL13. UL13 and ICP22 are also required for efficient viral transcription in HEL 299 and HeLa S3 cells. IE, DE, and especially L gene transcription is reduced in 22/*n*199 and *d*UL13 infected HEL 299 and HeLa S3 cells. Neither UL13 nor ICP22 appear to be required for efficient cellular transcription shutoff. Since UL13 and ICP22 are required for RNAP II modifications, RNAP II_I may not be required for cellular transcription repression.

UL13 and ICP22 mutants display similar phenotypes in terms of cell type dependent growth differences, RNAP II large subunit phosphorylation changes, RNAP II localization into DNA replication compartments, viral transcription and cellular transcription. Our results support the hypothesis that ICP22 and UL13 may be acting in the same pathway that leads to these phenotypes.

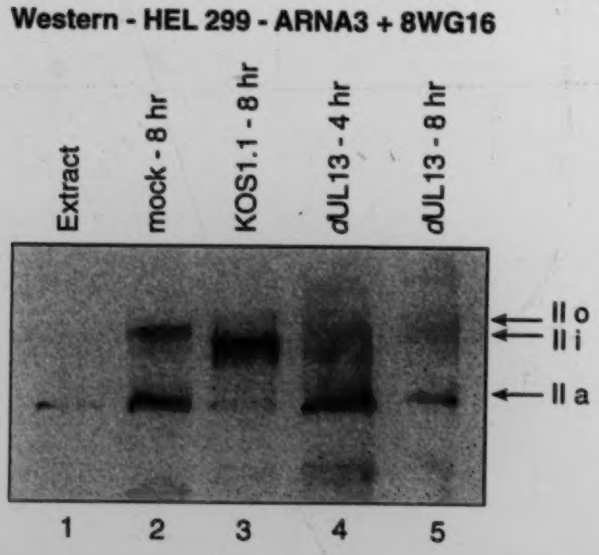
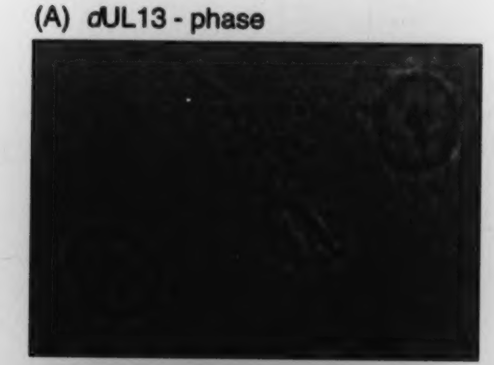

Western - HEL 299 - ARNA3 + 8WG16
Extract
mock - 8 hr
KOS1.1 - 8 hr
*d*UL13 - 4 hr
*d*UL13 - 8 hr
II o
II i
II a
1
2
3
4
5

Figure 5.1. Western blot analysis of the RNAP II large subunit in HSV-1 UL13 mutant *d*UL13 infected HEL 299 cells. HEL 299 cells were mock infected (lane 2) or infected with either WT HSV-1 (KOS1.1) (lane 3) or the HSV-1 UL13 mutant virus *d*UL13 (lanes 4 and 5) for the times indicated. Cells were harvested and lysed into Laemmli buffer. Extracts were run on SDS-PAGE (6% acrylamide) gels and subjected to immunoblotting. The samples were probed with a mixture of both RNAP II large subunit antibodies, ARNA3 and 8WG16. Each lane contained extract from the same number of cells, except lane 1, which contained an aliquot of a nuclear *in vitro* transcription extract prepared from HeLa cells. Lanes 1 to 3 are the same as those shown in Fig. 4.8.

Figure 5.2. Double immunofluorescence of RNAP II and ICP8 in HSV-1 UL13 mutant *d*UL13 infected Vero cells. Vero cells were infected with HSV-1 UL13 mutant *d*UL13 for 5 hr. The cells were fixed and stained with monoclonal antibody 8WG16 and anti-ICP8 polyclonal antiserum 3-83. Secondary antibodies were a mixture of rhodamine-conjugated goat anti-mouse immunoglobulin (for RNAP II visualization) and fluorescein conjugated donkey anti-rabbit immunoglobulin (for ICP8 visualization). Phase-contrast microscopy (A) of corresponding panels. Detection of RNAP II (B). Detection of ICP8 (C).

Double Immunfluorescence

(A) *d*UL13 - phase



(B) *d*UL13 - 8WG16

(C) *d*UL13 - 3-83

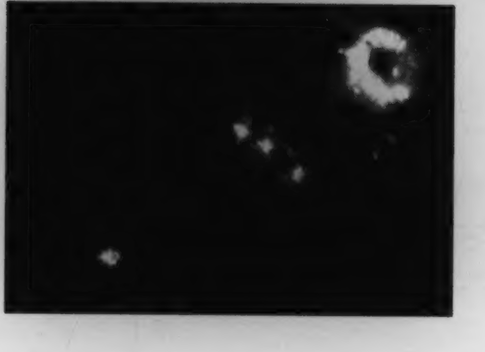


Figure 5.3. Nuclear run-on transcription analysis of viral gene transcription in infected Vero cells. Vero cells were infected with WT HSV-1 (KOS1.1) (A) or with the HSV-1 UL13 mutant *d*UL13 (B) for the times indicated. Nuclei were isolated and transcription was allowed to proceed in the presence of ^{32}P-UTP as described in Chapter 2. RNA products from equal numbers of nuclei per sample were hybridized to immobilized single-stranded DNA probes which detect sense (S) or antisense (AS) transcripts arising from the IE genes ICP4 and ICP27, the DE genes ICP8 and DNA polymerase, and the L genes VP16, gC, and UL36. Single-stranded DNA of M13mp19 was included as a background hybridization control. Nuclear run-on transcription assays of mock infected cells yielded no hybridization to these probes (data not shown).

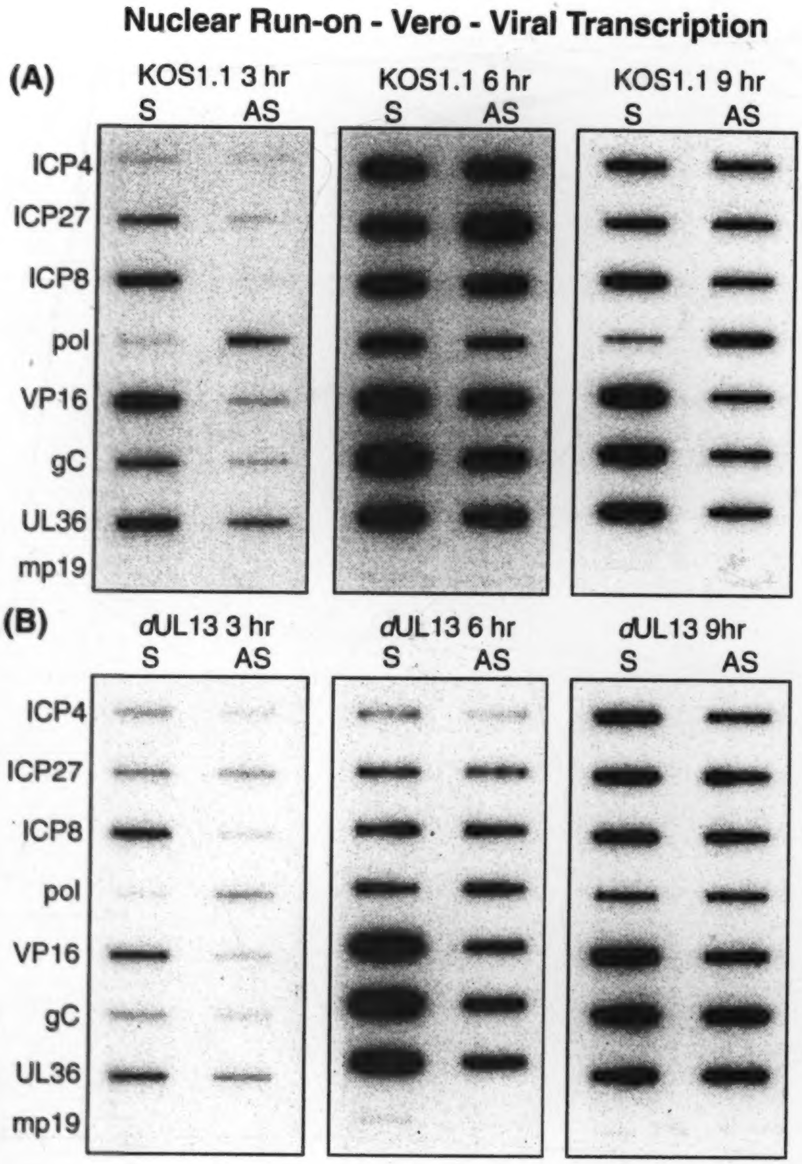
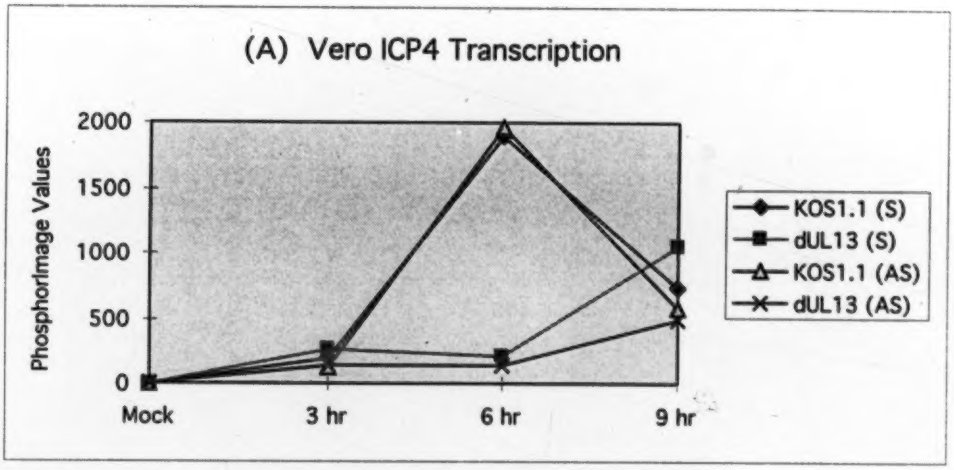
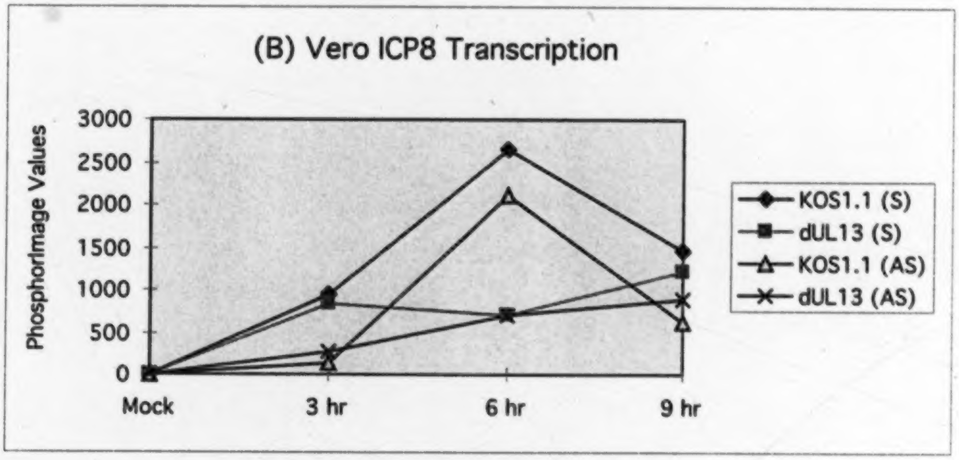

Nuclear Run-on - Vero - Viral Transcription
(A)
KOS1.1 3 hr
S AS
KOS1.1 6 hr
S AS
KOS1.1 9 hr
S AS
ICP4
ICP27
ICP8
pol
VP16
gC
UL36
mp19
(B)
dUL13 3 hr
S AS
dUL13 6 hr
S AS
dUL13 9hr
S AS
ICP4
ICP27
ICP8
pol
VP16
gC
UL36
mp19

Figure 5.4. Quantitation of viral gene transcription in infected Vero cells. The relative ^{32}P-hybridization signal to specific gene probes, detecting sense (S) or antisense (AS) transcripts, obtained from nuclear run-on analysis of viral transcription in Vero cells was quantitated by PhosphorImager analysis: top, ICP4; middle, ICP8; bottom, gC. PhosphorImager analysis was performed using a Fujix BAS100 bio-imaging analyzer with MacBAS imaging software. The M13mp19 background control was subtracted from each of the values.


(A) Vero ICP4 Transcription


(B) Vero ICP8 Transcription

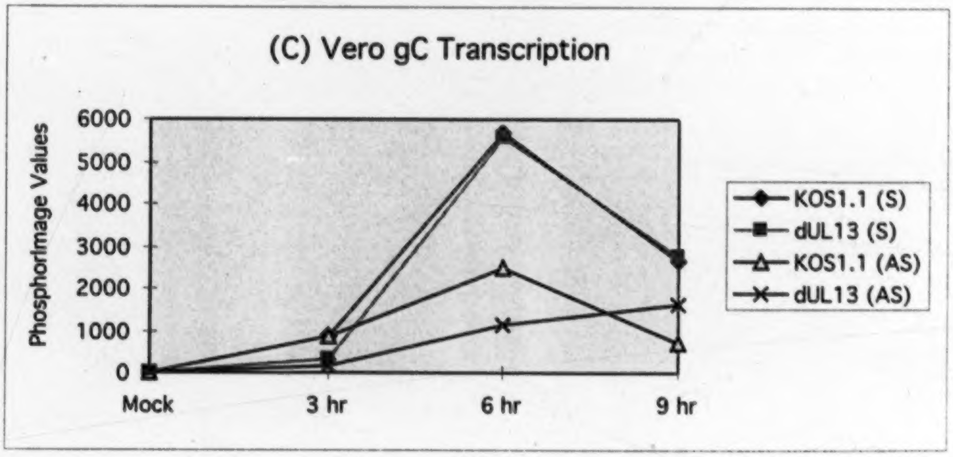

(C) Vero gC Transcription

Figure 5.5. Nuclear run-on transcription analysis of viral gene transcription in infected HeLa S3 cells. HeLa S3 cells were infected with WT HSV-1 (KOS1.1) (A) or with the HSV-1 UL13 mutant *d*UL13 (B) for the times indicated. Nuclear run-on transcription assays were performed as described in the legend to Fig. 5.3. Nuclear run-on transcription assays of mock infected cells yielded no hybridization to these probes (data not shown).

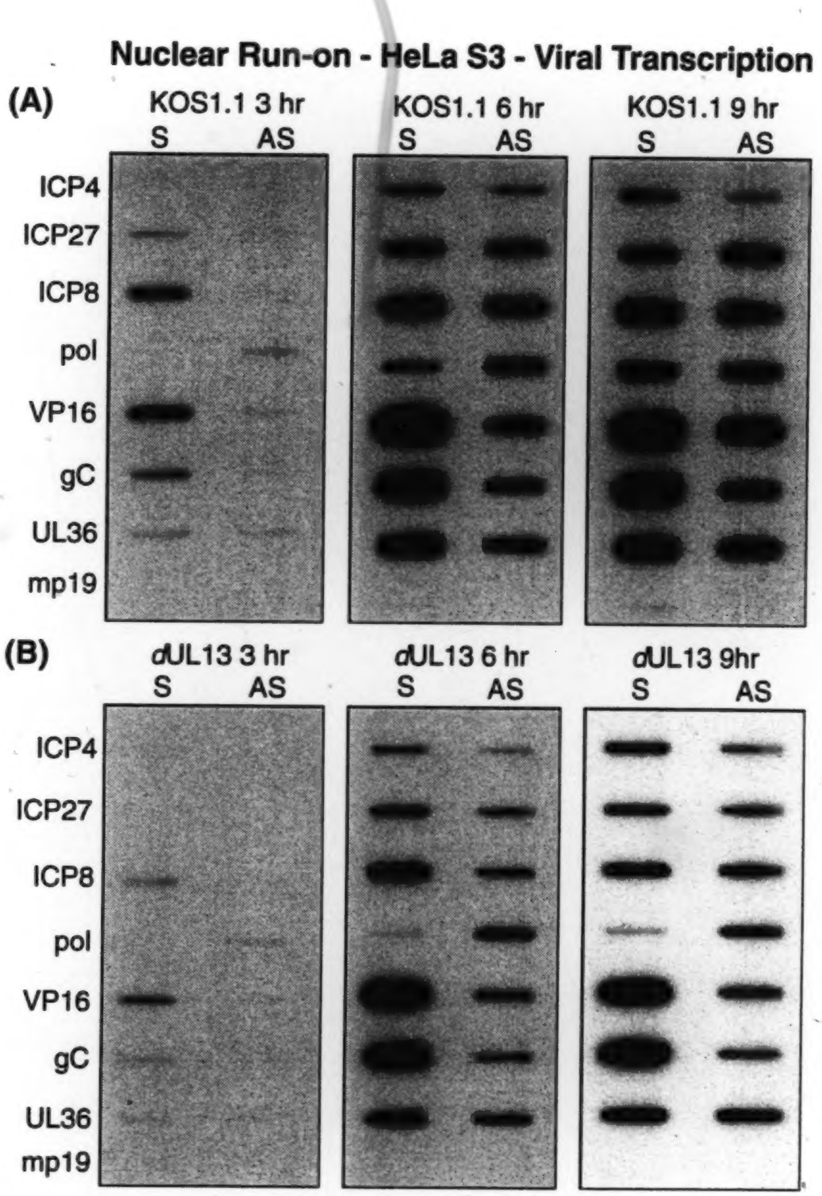
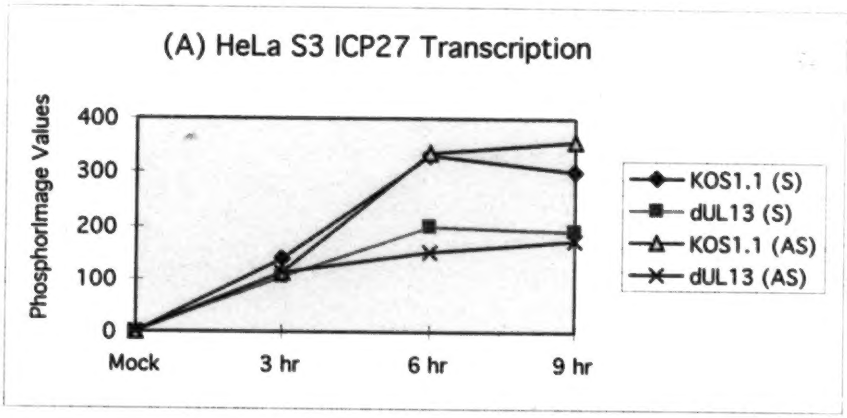
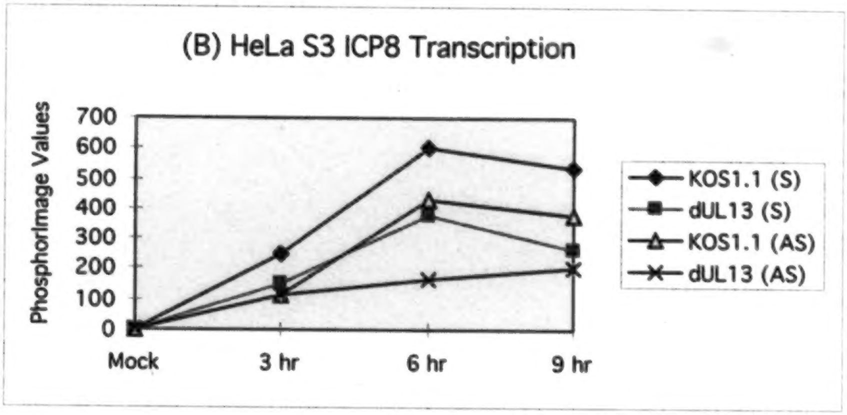

Nuclear Run-on - HeLa S3 - Viral Transcription
(A)
KOS1.1 3 hr
S
AS
KOS1.1 6 hr
S
AS
KOS1.1 9 hr
S
AS
ICP4
ICP27
ICP8
pol
VP16
gC
UL36
mp19
(B)
dUL13 3 hr
S
AS
dUL13 6 hr
S
AS
dUL13 9hr
S
AS
ICP4
ICP27
ICP8
pol
VP16
gC
UL36
mp19

Figure 5.6. Quantitation of viral gene transcription in infected HeLa S3 cells. The relative ^{32}P-hybridization signal to specific gene probes, detecting sense (S) or antisense (AS) transcripts, obtained from nuclear run-on analysis of viral transcription in HeLa S3 cells was quantitated by PhosphorImager analysis as described in the legend to Fig. 5.4: top, ICP27; middle, ICP8; bottom, VP16.


(A) HeLa S3 ICP27 Transcription
Phosphorlmage Values
400
300
200
100
0
Mock
3 hr
6 hr
9 hr
KOS1.1 (S)
dUL13 (S)
KOS1.1 (AS)
dUL13 (AS)


(B) HeLa S3 ICP8 Transcription
Phosphorlmage Values
700
600
500
400
300
200
100
0
Mock
3 hr
6 hr
9 hr
KOS1.1 (S)
dUL13 (S)
KOS1.1 (AS)
dUL13 (AS)

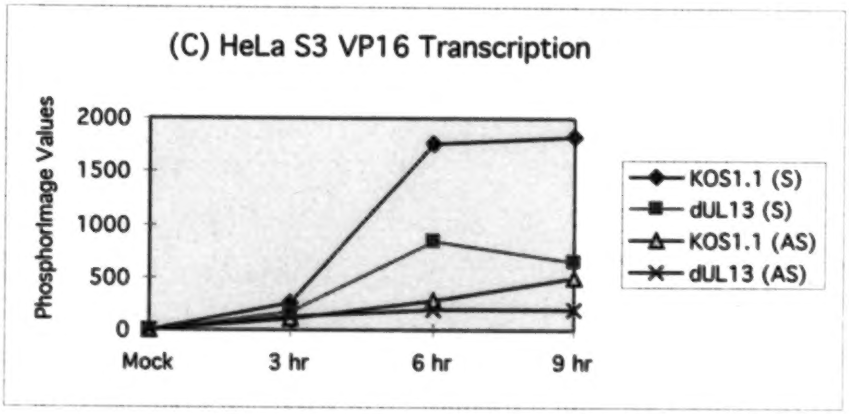

(C) HeLa S3 VP16 Transcription
Phosphorlmage Values
2000
1500
1000
500
0
Mock
3 hr
6 hr
9 hr
KOS1.1 (S)
dUL13 (S)
KOS1.1 (AS)
dUL13 (AS)

Figure 5.7. Nuclear run-on transcription analysis of viral gene transcription in HEL 299 infected cells. HEL 299 cells were infected with WT HSV-1 (KOS1.1) (A), the UL13 mutant *d*UL13 (B), or the ICP22 mutant 22/*n*199 (C) for the times indicated. Nuclear run-on transcription assays were performed as described in the legend to Fig. 5.3. Nuclear run-on transcription assays of mock infected cells yielded no hybridization to these probes (data not shown).

Nuclear Run-on - HEL 299 - Viral Transcription

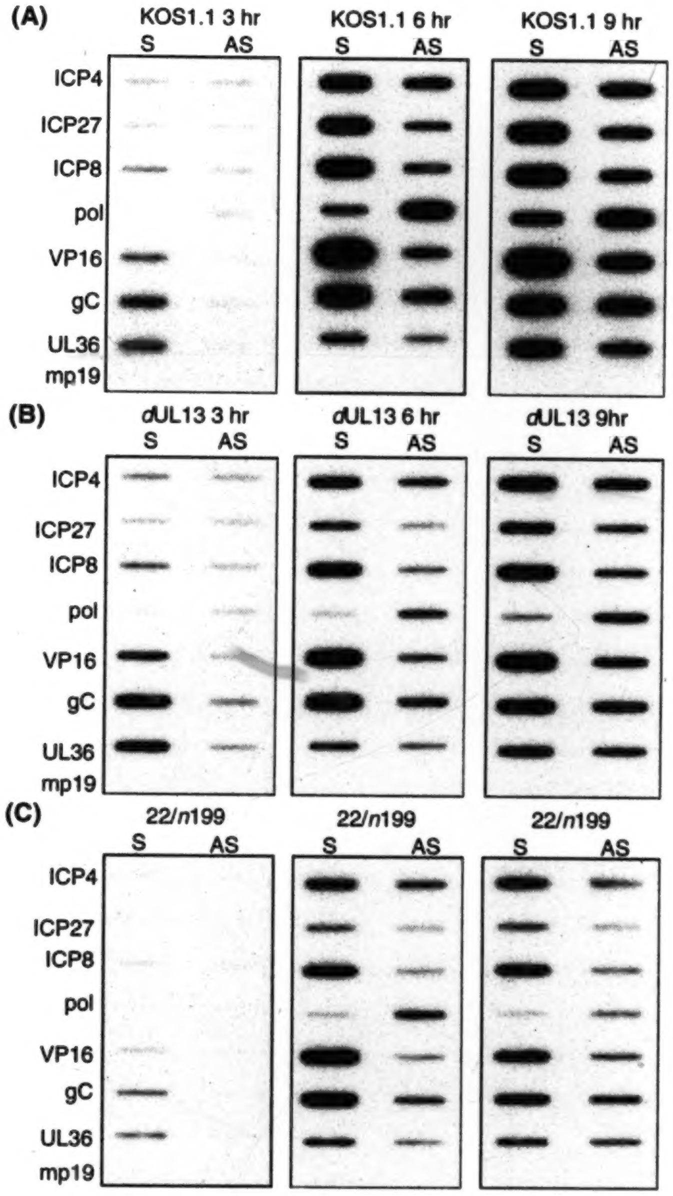
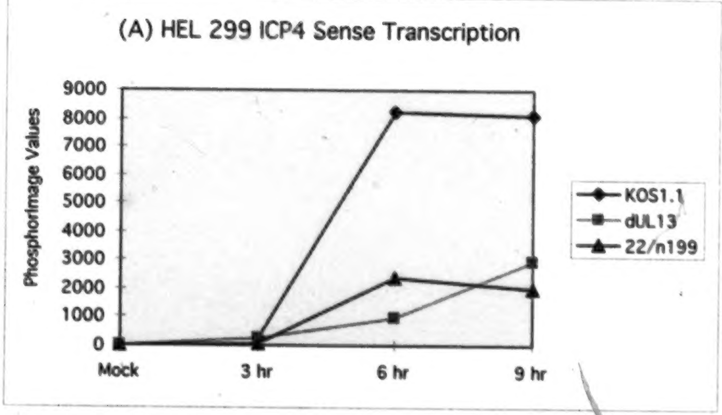
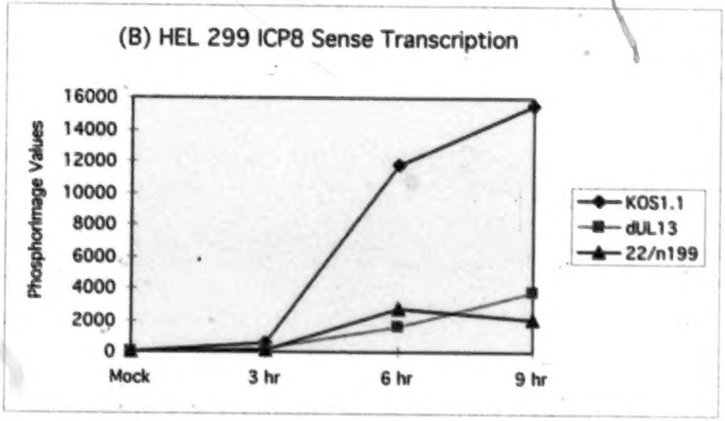

(A)
KOS1.1 3 hr
KOS1.1 6 hr
KOS1.1 9 hr
S
AS
ICP4
ICP27
ICP8
pol
VP16
gC
UL36
mp19
(B)
dUL13 3 hr
dUL13 6 hr
dUL13 9hr
(C)
22/n199
22/n199
22/n199

Figure 5.8. Quantitation of sense (S) viral gene transcription in infected HEL 299 cells. The relative ^{32}P-hybridization signal to specific sense (S) gene probes obtained from nuclear run-on analysis of viral transcription in HEL 299 cells is quantitated by PhosphorImager analysis as described in the legend to Fig. 5.4: top, ICP4; middle, ICP8; bottom, VP16.


(A) HEL 299 ICP4 Sense Transcription


(B) HEL 299 ICP8 Sense Transcription

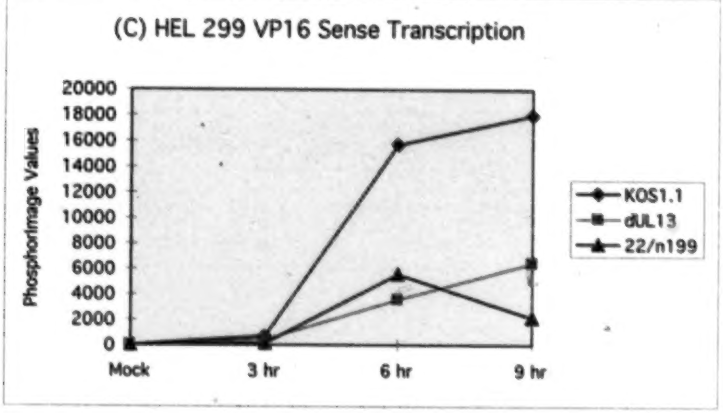
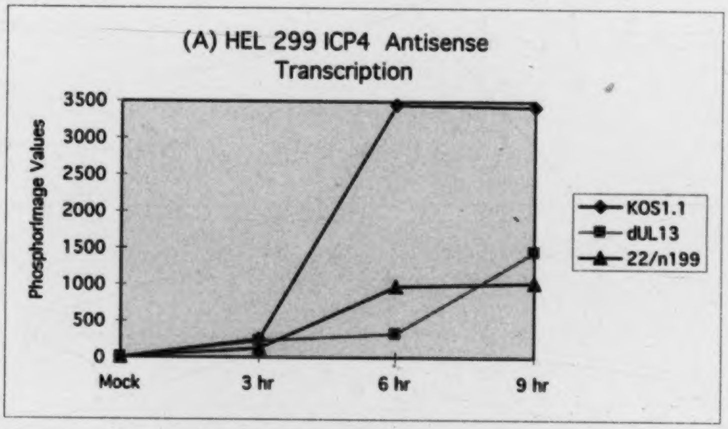
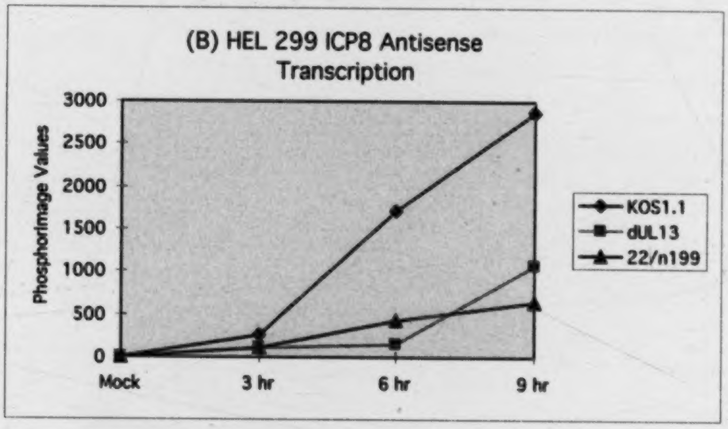

(C) HEL 299 VP16 Sense Transcription

Figure 5.9. Quantitation of antisense (AS) viral transcription in infected HEL 299 cells. The relative ^{32}P-hybridization signal to specific antisense (AS) probes obtained from nuclear run-on analysis of viral transcription in HEL 299 cells is quantitated by PhosphorImager analysis as described in the legend to Fig. 5.4: top, ICP4; middle, ICP8; bottom, VP16.


(A) HEL 299 ICP4 Antisense Transcription


(B) HEL 299 ICP8 Antisense Transcription

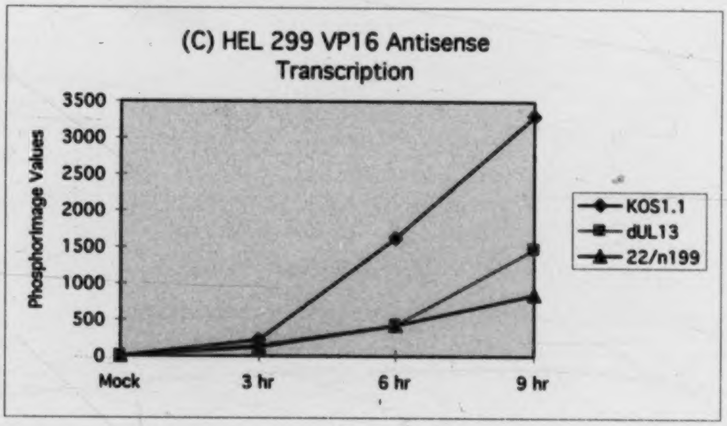

(C) HEL 299 VP16 Antisense Transcription

Figure 5.10. Nuclear run-on transcription analysis of cellular gene transcription in infected HeLa S3 cells. HeLa S3 cells were mock infected (A) or infected with WT HSV-1 (KOS1.1) (B) or the HSV-1 UL13 mutant *d*UL13 (C) for the times indicated. Nuclear run-on transcription assays were performed as described in the legend to Fig. 5.3 except that the filters contained single-stranded M13 DNA probes which detect sense (S) or antisense (AS) transcripts arising from the human c-myc, c-fos, γ-actin, GAPDH and histone H2B genes.

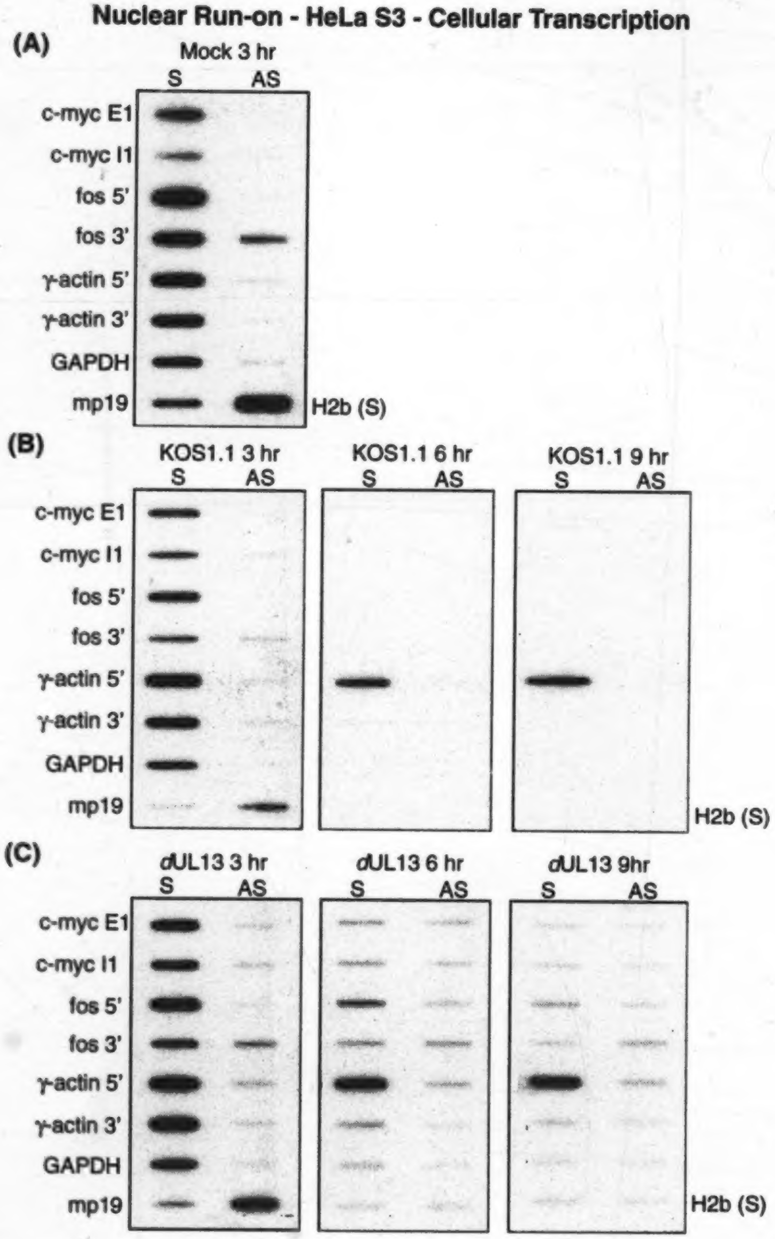

Nuclear Run-on - HeLa S3 - Cellular Transcription
(A)
Mock 3 hr
S
AS
c-myc E1
c-myc I1
fos 5'
fos 3'
γ-actin 5'
γ-actin 3'
GAPDH
mp19
H2b (S)
(B)
KOS1.1 3 hr
S
AS
KOS1.1 6 hr
S
AS
KOS1.1 9 hr
S
AS
c-myc E1
c-myc I1
fos 5'
fos 3'
γ-actin 5'
γ-actin 3'
GAPDH
mp19
H2b (S)
(C)
dUL13 3 hr
S
AS
dUL13 6 hr
S
AS
dUL13 9hr
S
AS
c-myc E1
c-myc I1
fos 5'
fos 3'
γ-actin 5'
γ-actin 3'
GAPDH
mp19
H2b (S)

TABLE 5.1 Quantitative comparison of transcription on cellular genes following 9 hr infection with WT HSV-1 (KOS1.1) and *d*UL13 virus in HeLa S3 cells[a]

GENE	KOS1.1	UL13$^-$
c-myc E1	0.8	3.6
c-myc I1	8.6	6.1
c-fos 5′	2.6	5.5
c-fos 3′	3.8	5.1
γ-actin 5′	51.2	68.9
γ-actin 3′	3.8	9.7
GAPDH	3.0	12.0
H2b	0.3	5.2

[a] Intensities of nuclear run-on transcription signals were determined by phosphorimage analysis. The levels of transcription are expressed as percent of mock, after subtracting background hybridization to M13 single-stranded DNA.

Figure 5.11. Graphic representation of data from Table 5.1. Cellular transcription levels in WT HSV-1 (KOS1.1) or UL13 mutant *d*UL13 infected HeLa S3 cells are expressed as percent of mock levels, after subtracting background hybridization to M13mp19 single-stranded DNA.

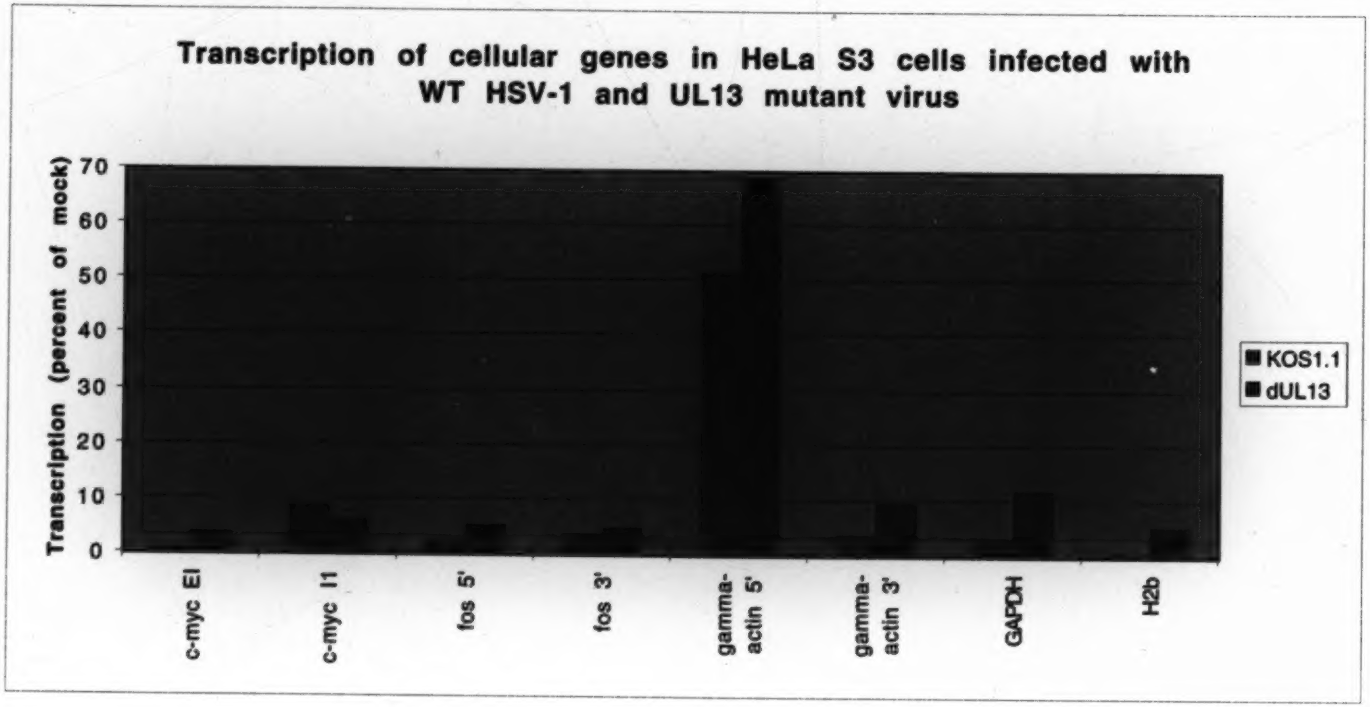
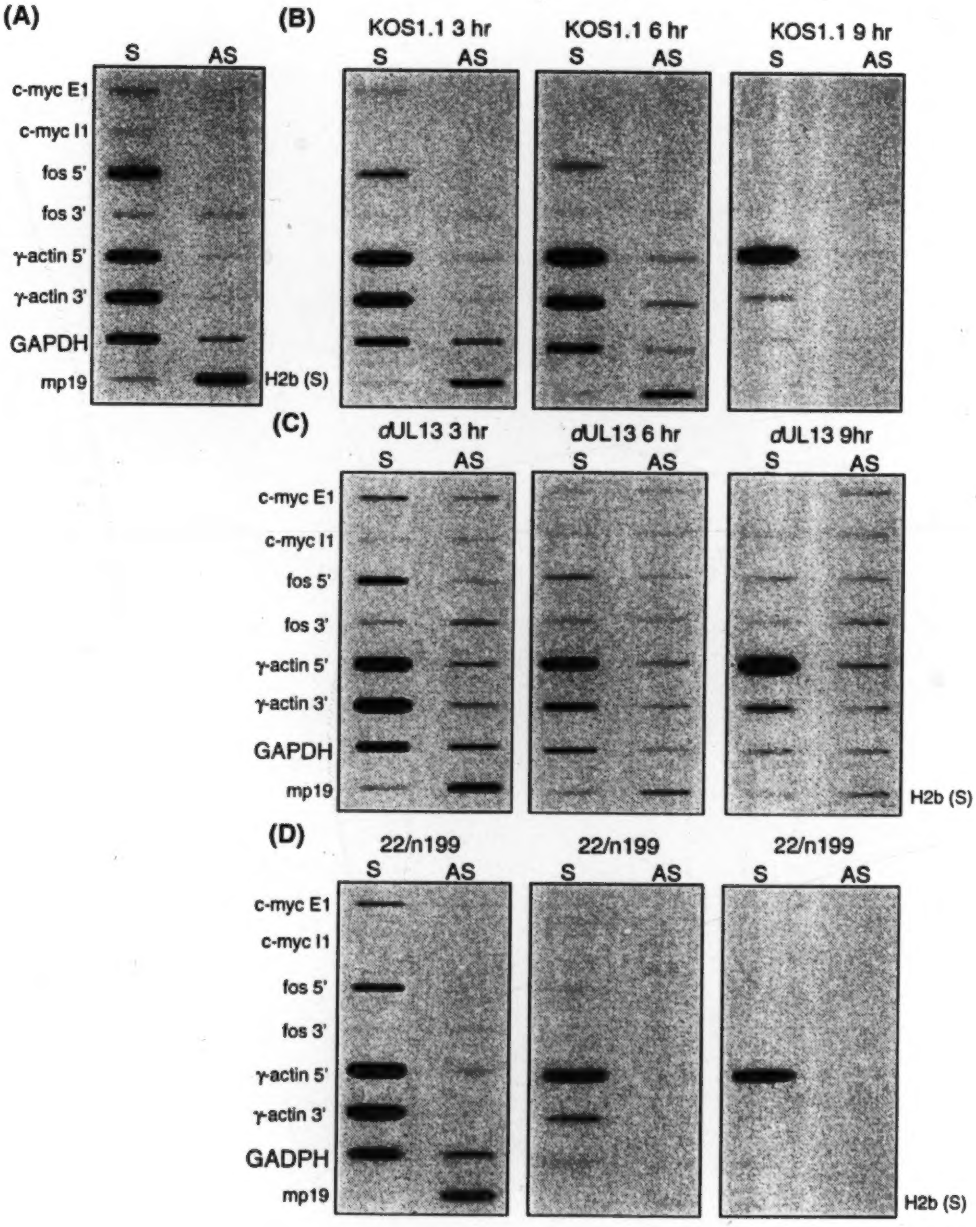

Transcription of cellular genes in HeLa S3 cells infected with WT HSV-1 and UL13 mutant virus
Transcription (percent of mock)
0
10
20
30
40
50
60
70
c-myc EI
c-myc I1
fos 5'
fos 3'
gamma-actin 5'
gamma-actin 3'
GAPDH
H2b
KOS1.1
dUL13

Figure 5.12. Nuclear run-on transcription analysis of cellular gene transcription in infected HEL 299 cells. HEL 299 cells were mock infected (A) or infected with WT HSV-1 (KOS1.1) (B), the UL13 mutant *d*UL13 (C), or the ICP22 mutant 22/*n*199 (D) for the times indicated. Nuclear run-on transcription assays were performed as described in the legend to Fig. 5.3 except that the filters contained single-stranded M13 DNA probes which detect sense (S) or antisense (AS) transcripts arising from the human c-myc, c-fos, γ-actin, GAPDH and histone H2B genes.



Nuclear Run-on - HEL 299 - Cellular Transcription

(A)
S
AS
c-myc E1
c-myc I1
fos 5'
fos 3'
γ-actin 5'
γ-actin 3'
GAPDH
mp19
H2b (S)
(B)
KOS1.1 3 hr
S
AS
KOS1.1 6 hr
S
AS
KOS1.1 9 hr
S
AS
(C)
dUL13 3 hr
S
AS
dUL13 6 hr
S
AS
dUL13 9hr
S
AS
c-myc E1
c-myc I1
fos 5'
fos 3'
γ-actin 5'
γ-actin 3'
GAPDH
mp19
H2b (S)
(D)
22/n199
S
AS
22/n199
S
AS
22/n199
S
AS
c-myc E1
c-myc I1
fos 5'
fos 3'
γ-actin 5'
γ-actin 3'
GADPH
mp19
H2b (S)

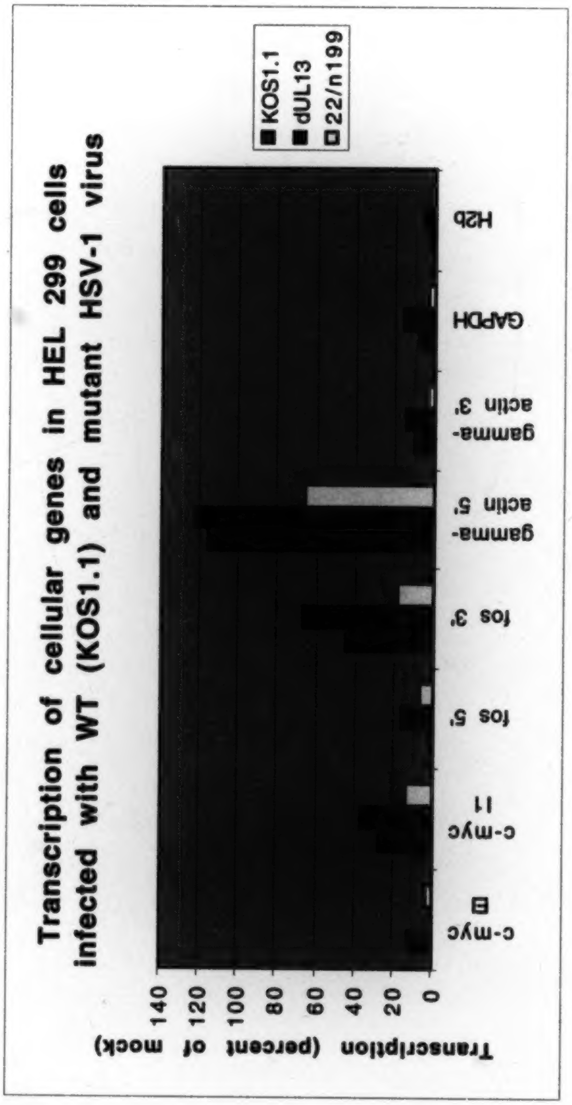
TABLE 5.2. Quantitative comparison of transcription on cellular genes following 9 hr infection with WT HSV-1 (KOS1.1), *d*UL13 and 22/*n*199 virus in HEL 299 cells[a]

GENE	KOS1.1	UL13$^-$	ICP22$^-$
c-myc E1	12.9	0.6	3.4
c-myc I1	28.5	37.6	14.0
c-fos 5′	2.4	16.2	6.8
c-fos 3′	45.6	68.0	18.4
γ-actin 5′	116.8	123.1	66.1
γ-actin 3′	10.0	15.0	3.5
GAPDH	8.5	16.8	4.0
H2b	3.6	6.4	2.7

---

[a] Intensities of nuclear run-on transcription signals were determined by phosphorimage analysis. The levels of transcription are expressed as percent of mock, after subtracting background hybridization to M13 single-stranded DNA.

Figure 5.13. Graphic representation of data from Table 5.2. Cellular transcription levels in WT HSV-1 (KOS1.1), UL13 mutant *d*UL13, or ICP22 mutant 22/*n*199 infected HEL 299 cells are expressed as percent of mock levels, after subtracting background hybridization to M13mp19 single-stranded DNA.


Transcription of cellular genes in HEL 299 cells infected with WT (KOS1.1) and mutant HSV-1 virus
Transcription (percent of mock)
0
20
40
60
80
100
120
140
c-myc E
c-myc I1
fos 5'
fos 3'
gamma-actin 5'
gamma-actin 3'
GAPDH
H2b
KOS1.1
dUL13
22/n199

CHAPTER SIX. THE EFFECTS OF HSV-1 INFECTION ON BASAL TRANSCRIPTION FACTORS AND PUTATIVE CTD KINASES

A. Introduction

An important but unresolved question is how the host's RNAP II transcription apparatus is directed to viral genes after HSV-1 infection (Smiley et al., 1991; Martin and Green, 1992). HSV-1 may accomplish the switch from cellular to viral transcription by altering the RNAP II transcription machinery in such a manner that allows the viral genome to be preferentially transcribed. Consistent with this hypothesis, we have found that HSV-1 infection results in two modifications to RNAP II. Firstly, the large subunit of RNAP II is rapidly and aberrantly phosphorylated following infection. Secondly, RNAP II is recruited into DNA replication compartments (Chapter 3).

The efficient modification of RNAP II requires the IE protein ICP22 and the viral kinase UL13 (Chapters 4 and 5). The induction of the IIi form after HSV-1 infection may not only depend on these two viral proteins but may also require the aberrant activity of cellular CTD kinases. CTD kinases are operationally defined as protein kinases that phosphorylate the consensus heptapeptide repeat of the CTD and cause the characteristic mobility shift of IIa to IIo on SDS-PAGE gels (Dahmus, 1994; Kang and Dahmus, 1995). Phosphorylation on the CTD has been found on serine, threonine (Chapter 3; Cadena and Dahmus, 1987; Zhang and Corden, 1991a), and tyrosine residues (Baskaran et al., 1993). This observation suggests that more than one CTD kinase is involved in CTD phosphorylation (Dahmus, 1994).

Putative CTD kinases have been characterized mainly *in vitro* by their ability to phosphorylate synthetic peptides corresponding to the consensus heptapeptide repeats, recombinant CTD or native RNAP IIa (Dahmus, 1994). Whether these CTD kinases function *in vivo* is unclear. Putative mammalian CTD kinases which are suspected to act *in vivo* include: TFIIH (Lu et al., 1992; Serizawa et al., 1995; Shiekhattar et al., 1995), DNA-dependent protein kinase (DNA-PK) (Arias et al., 1989; Anderson and Lees-Miller, 1992; Dvir et al., 1992; Anderson et al., 1995), the cell cycle control protein $p34^{cdc2}$ (Cisek and Corden, 1989; Zawel et al., 1993), the protein encoded by the c-abl proto-oncogene (Baskaran et al., 1996), tat-associated kinase (TAK) (Herrmann and Rice, 1993; Herrmann and Rice, 1994), CTD-K1 and CTD-K2 (Payne and Dahmus, 1993), and the protein complex of cyclin C and cyclin-dependent kinase (CDK) 8 (Leclerc et al., 1996; Rickert et al., 1996). These putative CTD kinases are described in detail in Chapter 1.

By examining the CTD kinases after HSV-1 infection, we may be able to identify the kinases which are targeted by the virus. Those CTD kinases altered by viral infection may also be involved in modifying the large subunit of RNAP II. Furthermore, because most of the CTD kinases have only been identified *in vitro*, determining the kinases that are altered after HSV-1 infection may provide insight on which CTD kinase functions *in vivo* during the transcription process.

It is also possible that HSV-1 shifts RNAP II transcription from cellular to viral genes by targeting other members of the RNAP II basal transcription machinery, such as TBP, TAFs or other general transcription factors (GTFs). In this chapter, I describe experiments that analyze the abundance, localization, and modifications to members of the RNAP II basal transcription machinery following HSV-1 infection. These basal factors include 2 putative CTD kinases (MO15 subunit of TFIIH and CDK8), both of which are thought to form part of the RNAP II holoenzyme (Koleske and Young, 1995; Ossipow et al., 1995; Chao et al., 1996; Maldonado et al., 1995; Wilson et al., 1996).

B. TBP

TBP is a highly conserved protein, which plays a role in RNAP I, II, and III transcription. TBP is the TATA box binding protein of TFIID and is tightly associated with at least 12 TBP-associated factors (TAFs), ranging from 18 to 250 kDa (Goodrich and Tjian, 1996; Goodrich et al., 1996, Verrijzer and Tjian, 1996; Tansey and Herr, 1997). According to the multistep model of preinitiation complex (PIC) assembly, the first step forming the PIC is the binding of TBP to the TATA box (reviewed in Hernandez, 1993).

Modifications to TBP have been previously described (Jackson and Tjian, 1988; Ohkuma and Roeder, 1994; Sweillens and Pirson, 1994). Recent studies suggest that TBP may be modified by glycosylation (Bustamante et al., 1995) and phosphorylation. TBP is phosphorylated by TFIIH *in vitro*. This phosphorylation is enhanced by the 56 kDa subunit of TFIIE and occurs on serine (34%) and threonine (66%) residues from amino acids 1 to 154 (Ohkuma and Roeder, 1994), a region which resembles the heptapeptide consensus sequences of the CTD. The effects of TBP phosphorylation are unclear. TBP phosphorylation may influence protein-protein interactions between TBP and another basal or activated transcription factor and as a result may affect basal and activator dependent transcription initiation.

TBP protein levels are constant throughout the cell cycle, but the phosphorylation state of TBP varies. During mitosis, TBP becomes hyperphosphorylated. Asynchronous populations of cells contain mainly nonphosphorylated TBP (White et al., 1995; Parsons and Spencer, 1997). Phosphorylated TBP is not readily detected in these extracts as mitotic cells only make up approximately 4% of the asynchronous population (Terasima and Tolmach, 1963).

To test whether TBP was altered (i.e. phosphorylation or protein abundance) by HSV-1 infection, I analyzed TBP by Western blotting. Vero cells were mock infected or infected with HSV-1 (KOS1.1) for various times, harvested, and lysed into Laemmli buffer. Total proteins were separated on SDS-PAGE gels and Western blotting was conducted using TBP18, an antibody recognizing TBP (Fig. 6.1). Mock infected Vero cells contained TBP, migrating at approximately 38 kDa (Fig. 6.1, lane 1). The extracts were prepared from an asynchronous population of cells and TBP would be expected to be mostly nonphosphorylated (White et al., 1995). Infection of KOS1.1 resulted in generally constant levels of TBP protein throughout infection (Fig. 6.1, lanes 2 to 6). Furthermore, after infection, there were no detectable changes in the migration of the TBP, suggesting that the phosphorylation state of TBP is not altered after infection.

We then wanted to determine if the absence of TBP alterations after infection was specific to Vero cells or if this also occurred in another cell line. Western blotting experiments using TBP18 were performed with HeLa S3 extracts infected with HSV-1 (data not shown). Similar results to that observed in Vero cells were found with HeLa S3 cells. After HSV-1 infection in Vero and HeLa S3 cells, the migration or the abundance of TBP did not appear to be altered.

To determine the nuclear distribution of TBP during HSV-1 infection, we performed indirect immunofluorescence experiments (data not shown). Vero cells were mock infected or infected with HSV-1 (KOS1.1) for 5 hr. The cells were subsequently fixed and stained with TBP18. In mock infected cells, TBP staining displayed a diffuse uniform nuclear distribution, with pronounced exclusion from the nucleoli, sites of RNAP I transcription. Surprisingly TBP, a "universal" transcription factor that plays a role in RNAP I, II and III transcription (Hernandez, 1993), was excluded from the nucleolus. After infection with HSV-1, the majority of TBP was localized into a number of discrete subnuclear foci. As infection proceeded, these compartments fused into larger globules which filled up most of the nucleus (data not shown).

We then asked whether the sites of TBP localization in infected cells were identical to DNA replication compartments. We performed double immunofluorescence experiments to see if TBP colocalized with ICP8, the major HSV-1 DNA binding protein which associates with DNA replication compartments (Quinlan et al., 1984). Vero cells were infected with HSV-1 for 5 hr, fixed, and then stained with primary antibodies TBP18 and 3-83, an antiserum directed against ICP8. Immunofluorescence staining patterns for TBP and ICP8 after infection were coincident (Fig. 6.2), indicating that TBP is preferentially localized and recruited into DNA replication compartments after HSV-1 infection.

A series of control experiments verified the above results. There was no cross reaction of the TBP and ICP8 antibodies and of the two fluorescence secondary antibodies (data not shown). ICP8 showed some peripheral staining in the nucleus and also in the cytoplasm (Fig. 6.2C), which did not colocalize with TBP staining (Fig. 6.2B). This was also observed previously in where ICP8 showed peripheral staining in the nucleus and in the cytoplasm (Fig. 3.2B and 3.2D) which did not colocalize with RNAP II staining (Fig. 3.2A and 3.2C). This particular staining pattern may be due to a specific interaction between rabbit immunoglobulins and an Fc receptor encoded by HSV-1 (Rice, Unpublished data; Johnson et al., 1988). This staining provided us with an internal control for the lack of cross-reaction between the TBP18 and 3-83 primary antibodies.

The data demonstrate that after HSV-1 infection, the protein levels and phosphorylation state of TBP are not altered as detected by Western blot. Furthermore, TBP and ICP8 colocalize into DNA replication compartments. After infection, TBP, along with RNAP II (Chapter 3), is shuttled from a disperse distribution within the nucleus to viral replication compartments. These results suggest that DNA replication compartments may serve as sites for viral transcription.

C. TFIIB

According to the multistep model of PIC assembly, TFIIB binds to TBP-DNA complexes and aids in the recruitment of RNAP II and TFIIF to the PIC (Zawel and Reinberg, 1993; Zawel and Reinberg, 1995; Orphanides et al., 1996). In addition, TFIIB may play a role in RNAP II transcription start site selection. Mutations in the yeast TFIIB gene alter the position of RNAP II initiation (Orphanides et al., 1996). To date, TFIIB has not been found to be a modified posttranslationally by either phosphorylation or glycosylation.

To test whether TFIIB protein levels were altered after HSV-1 infection, I analyzed TFIIB by Western blotting. The immunoblot from Fig. 6.1 containing samples of Vero cells that were mock infected or infected with HSV-1 (KOS1.1) for various times, was stripped and reprobed with α–TFIIB, an affinity purified polyclonal antibody which specifically recognizes TFIIB (Fig. 6.3). Mock infected Vero cells contained TFIIB which migrated at approximately 35 kDa (Fig. 6.3, lane 1). Infection with KOS1.1 from 1 to 4 hr resulted in no gross changes to TFIIB protein levels (Fig. 6.3, lanes 2 to 4). However, at 5 and 8 hr of infection, TFIIB protein abundance appeared to decline (Fig. 6.3, lanes 5 and 6). The decreases in TFIIB protein levels may be authentic and not due to differences in protein loading (or cell number) in each of the lanes on the gel, since TBP protein levels did not decline after HSV-1 infection (Fig. 6.1). There were some faster migrating products observed below the TFIIB band, which may be either degradation products of the TFIIB protein or proteins (viral or cellular) which cross-react with the α–TFIIB antibody. Similar bands were observed in mock infected samples in other Western blotting experiments using α–TFIIB (data not shown), even though they are not observed in the mock infected sample in Fig. 6.3.

I then wanted to determine if TFIIB alterations observed late in infection were specific to Vero cells or also occurred in another cell line. Western blotting experiments using α–TFIIB antibody were performed with HeLa S3 extracts infected with HSV-1 (data not shown). Similar results were found with HeLa S3 cells, as observed in Vero cells, where after infection, TFIIB protein levels declined. TFIIB protein levels also decline after infection of HeLa S3 and HEL 299 cells over an 9 hr time period (Kilvert, 1995, Unpublished data). These data suggests that TFIIB abundance is altered after HSV-1 infection in several cell types.

I attempted to examine the distribution of TFIIB during HSV-1 infection by indirect immunofluorescence studies. The results from these experiments were inconclusive because α–TFIIB immunofluorescence staining was largely nonspecific in both mock infected and KOS1.1 infected cells. The α–TFIIB antibody appeared to cross-react with other proteins and bright staining was observed throughout the cytoplasm and nucleus (data not shown).

D. TFIIE

TFIIE is a tetramer containing two p34 and two p56 subunits (Inostroza et al., 1991; Orphanides et al., 1996). According to the multistep model of PIC assembly, TFIIE recruits TFIIH to the PIC (Flores et al., 1992; Maxon et al., 1994) and regulates TFIIH kinase and helicase activities (Drapkin et al., 1994a; Okhuma and Roeder, 1994). TFIIE may play a direct or indirect role in promoter melting (reviewed in Orphanides et al., 1996; Roeder, 1996). In addition, TFIIE is also a component of most RNAP II holoenzymes (Ossipow et al., 1995; Chao et al., 1996; Maldonado et al., 1996).

The 56 kDa subunit of TFIIE (p56) is modified by phosphorylation in interphase cells. When the cells progress to mitosis, up to 50% of p56 protein is hyperphosphorylated (Parsons and Spencer, 1997). *In vitro* phosphorylation assays with purified transcription factors show that p56 is phosphorylated by TFIIH on serine residues and that this is enhanced by the 34 kDa subunit of TFIIE (p34). At present, the significance of this phosphorylation is unclear. The phosphorylation of p56 could affect protein-protein interactions in the formation of the PIC and, ultimately, affect basal or activated transcription (Ohkuma and Roeder, 1994).

To test whether the p56 was altered (i.e. phosphorylation, protein level changes) after HSV-1 infection, I analyzed p56 by Western blotting. HeLa S3 cells were mock infected or infected with HSV-1 (KOS1.1) for various times. Cell extracts were prepared and total proteins were separated on SDS-PAGE gels. Western blotting was performed using α–p56E, an affinity purified polyclonal antibody directed against p56 of human TFIIE (Fig. 6.4).

Western blot analysis showed that mock infected HeLa S3 cells contained p56, migrating at approximately 65 kDa (Fig. 6.4, lane 1). Infection of KOS1.1 did not alter the levels of p56 protein up to 8 hr post-infection (Fig. 6.4, lanes 2 and 4). In lane 3, there appeared to be a loss of p56 protein, which we suspect may be due to a transfer problem during Western blotting. Levels of p56 also remain constant after HSV-1 infection of Vero and HEL 299 cells (Kilvert, 1995, Unpublished data). No alterations in the migration of p56 were detected, suggesting that the phosphorylation state of p56 remains constant after infection.

The distribution of p56 during HSV-1 infection was examined by indirect immunofluorescence (Kilvert, 1995, Unpublished data). In mock infected cells, p56 staining is nuclear with exclusion from the nucleoli. After KOS1.1 infection, there is bright specific staining of p56 within the DNA replication compartments. However, moderate levels of background staining are also present throughout the nucleus. These results indicate that p56 is localized to DNA replication compartments after infection, but the recruitment of the protein into these subnuclear foci may not be as efficient as with RNAP II (Chapter 3) and TBP (this chapter). Alternatively, the α–p56E antibody may cross-react with other nuclear proteins in immunofluorescence assays.

The data suggests that after HSV-1 infection, the p56 subunit of TFIIE is not altered in abundance or phosphorylation, as detected by Western blotting. The p56 protein appears to localize, to some extent, into DNA replication compartments after HSV-1 infection (Kilvert, 1995, Unpublished data). Staining with the α–p56E antibody detects some additional staining outside the viral replication compartments, which may or may not be genuine p56 signal.

E. TFIIF

TFIIF is composed of two subunits, RAP30 and RAP74. According to the multistep model of PIC assembly, TFIIF binds to RNAP II and delivers RNAP II to the DAB complex on DNA. RAP30 is capable of independently recruiting RNAP II to the DAB complex but the DAB-RNAP II/RAP30 complex is less stable without RAP74. This suggests that RAP74 stabilizes the DAB-RNAP II/TFIIF complex (Zawel and Reinberg, 1993; Zawel and Reinberg, 1995; Orphanides et al., 1996). In addition to its initiation functions, TFIIF stimulates elongation (Reines et al., 1996). TFIIF has also been implicated in transcription initiation site selection (Orphanides et al., 1996) and is a member of most RNAP II holoenzymes (Ossipow et al., 1995; Chao et al., 1996; Maldonado et al., 1996).

TFIIF is extensively phosphorylated *in vivo* on the RAP74 subunit. Experiments conducted by Kitajima et al. (1994) show that phosphorylation of TFIIF greatly affects the protein's function, such that transcription initiation and elongation stimulating activities decrease when TFIIF is treated with alkaline phosphatase. Furthermore, phosphatase treated TFIIF has a reduced ability to bind to TFIIB and assemble into TFIID/TFIIB/RNAP II/TFIIF complexes. These results indicate that TFIIF activity may be regulated by protein phosphorylation, particularly of the RAP74 subunit (Kitajima et al., 1994). *In vitro* experiments with purified transcription factors demonstrate that RAP74 is phosphorylated by TFIIH (Ohkuma and Roeder, 1994) and $TAF_{II}250$ (Dikstein et al., 1996). The phosphorylation of RAP74 by TFIIH is independent of the presence of RAP30 or TFIIE (Ohkuma and Roeder, 1994). $TAF_{II}250$ selectively phosphorylates RAP74 but not other basal transcription factors (Dikstein et al., 1996). These modifications may help modulate TFIIF function.

To test whether the RAP74 subunit of TFIIF was altered (i.e. phosphorylation, protein level changes) after HSV-1 infection, I analyzed RAP74 by Western blotting. The immunoblot from Fig. 6.4, containing samples of uninfected and HSV-1 (KOS1.1) infected HeLa S3 cells, was stripped and reprobed with α–RAP74, an affinity purified polyclonal antibody directed against RAP74 of human TFIIF (Fig. 6.5). Mock infected HeLa S3 cells contained RAP74, that migrated at approximately 78 kDa (Fig. 6.5, lane 1). This form of RAP74 is likely to be phosphorylated, as the protein is hyperphosphorylated *in vivo* and when purified from HeLa cells (Kitajima et al., 1994). Constant levels of RAP74 protein were present throughout KOS1.1 infection (Fig. 6.5, lanes 2 to 4). Levels of RAP74 are also unchanged after HSV-1 infection of Vero and HEL 299 cells (Kilvert, 1995, Unpublished data). After HSV-1 infection, the migration of RAP74 was not detectably altered on SDS-PAGE, suggesting that the phosphorylation state of RAP74 also remained constant.

The distribution of RAP74 during HSV-1 infection was examined by indirect immunofluorescence studies (Kilvert, 1995, Unpublished data). Mock infected cells showed RAP74 nuclear staining with exclusion from the nucleoli. After KOS1.1 infection, there was some specific staining of RAP74 in DNA replication compartments. However, there were also moderate to high levels of background staining present throughout the nucleus, as observed with the α–p56E antibody. Therefore, RAP74 is localized to DNA replication compartments after infection, but the recruitment of the protein to these subnuclear foci may not be as efficient as with RNAP II (Chapter 3) and TBP (this chapter). Alternatively, the α–RAP74 antibody may be cross-reacting with other nuclear proteins in immunofluorescence assays.

The data suggest that after HSV-1 infection, the RAP74 subunit of TFIIF is not altered by phosphorylation or by abundance, as detected by Western blotting. The RAP74 protein appears to preferentially localize to DNA replication compartments after HSV-1 infection (Kilvert, 1995, Unpublished data). The α–RAP74 antibody shows some additional nuclear staining outside the viral replication compartments, which may or may not be genuine RAP74 signal.

F. TFIIH

In the stepwise model, TFIIH is one of the last additions to the preinitiation complex (PIC) and is a multifunctional, multisubunit protein comprising of six or seven polypeptides between 35 and 89 kDa (Roeder, 1996; Svejstrup et al., 1996). In addition, TFIIH is a component of RNAP II holoenzymes (Koleske and Young, 1995; Ossipow et al., 1995; Chao et al., 1996; Maldonado et al., 1995; Wilson et al., 1996). We were particularly interested in examining whether there were any alterations to TFIIH after infection, because TFIIH contains a kinase activity that is capable of phosphorylating the CTD of RNAP II (Dahmus, 1994; Kang and Dahmus, 1995; Dahmus, 1996). TFIIH also possesses a bi-directional DNA helicase activity which may be required for promoter clearance (Serizawa et al., 1993; Roy et al., 1994).

In humans, the kinase moiety of TFIIH, capable of phosphorylating the CTD of RNAP II large subunit, consists of MO15 (CDK7), cyclin H, and MAT1 (p32). This tripartite complex also exists outside of TFIIH and is known as CDK activating kinase (CAK) (Clarke, 1995; Serizawa et al., 1995; Shiekhattar et al., 1995; Svejstrup et al., 1996). The levels of MO15 protein expression and CAK activity are constant throughout the eukaryotic cell cycle (Tassan et al., 1994). To date, no experiments have been performed to determine whether MO15 phosphorylation is required for TFIIH's CTD phosphorylation activity. It is possible that the activation of MO15 may play a role in the modulation of CTD phosphorylation and transcription *in vivo*.

To test whether the protein levels or phosphorylation state of the MO15 subunit of TFIIH were altered after HSV-1 infection, I analyzed MO15 by Western blotting. The immunoblot from Fig. 6.4 and 6.5, containing samples of uninfected and HSV-1 (KOS1.1) infected HeLa S3 cells, was stripped and reprobed with MO1.1, a monoclonal antibody directed against MO15 (Tassan et al., 1994) (Fig. 6.6). Western blot analysis showed that mock infected HeLa S3 cells contained MO15, migrating at approximately 40 kDa (Fig. 6.6, lane 1). It is likely that this band represents phosphorylated MO15, as CAK activity is constitutive in an asynchronous population of cells (Tassan et al., 1994) and is correlated with phosphorylation of threonine 176 and serine 170 (Fisher and Morgan, 1995). Infection with KOS1.1 resulted in declines in the levels of MO15 protein early in infection, between 0 and 2 hr (Fig. 6.6, lanes 1 and 2). The decline in MO15 protein levels may be authentic and not due to differences in protein loading (or cell number) between 0 and 2 hr, since p56 and RAP74 protein levels did not decline after HSV-1 infection (Fig. 6.4 and Fig. 6.5, lanes 1 and 2). After 2 hr, there were no gross changes in the levels of MO15 protein (Fig. 6.6, lanes 2 to 4). Furthermore, migration of MO15 on Western blots did not change after infection, suggesting that the phosphorylation state of MO15 remained constant.

The distribution of MO15 is nuclear at all stages of the cell cycle, except at mitosis, when the protein is present throughout the cell (Tassan et al., 1994). The localization of MO15 during HSV-1 infection was examined by indirect immunofluorescence studies (Kilvert, 1995, Unpublished data). MO15 was dispersed throughout the nucleus, with exclusion from the nucleoli, in mock infected Vero cells. After KOS1.1 infection, MO1.1 was recruited to DNA replication compartments (Kilvert, 1995, Unpublished data).

MAT1, another component of CAK, is a protein that does not fluctuate in abundance throughout the cell cycle. The protein migrates as a single band in HeLa S3 extracts (Tassan et al., 1995). Alison Kilvert has examined if HSV-1 infection results in alterations to MAT1 (1995, Unpublished data). Her results indicate that MAT1 protein levels do not change after infection in HEL 299, HeLa S3 and Vero cells.

I then attempted to examine the distribution of MAT1 by indirect immunofluorescence in HSV-1 infected HeLa CCL2.2 cells 5 hr post-infection. Staining of the cells was performed using the primary α–p32 antibody (generously provided by Dr. Erich Nigg, University of Geneva, Geneva, Switzerland), which recognizes MAT1 (Tassan et al., 1995), and the secondary antibody DARF. MAT1 staining was extremely weak (data not shown). Alison Kilvert repeated these experiments using α-rabbit biotinylated secondary antibody and α-streptavidin Texas Red instead of

DARF to detect MAT1 (1995, Unpublished data). In mock infected cells, MAT1 staining is nuclear, with exclusion from the nucleoli. This confirmed previous results observed by Tassan et al. (1995). After HSV-1 infection, some specific localization of MAT1 into DNA replication compartments was observed, but the results are somewhat inconclusive because background staining was high in the cytoplasm and the nucleus. The MAT1 antibody may be cross-reacting with other cellular and nuclear proteins in immunofluorescence assays.

We attempted to examine the protein levels of another subunit of TFIIH, p62, after HSV-1 infection. I analyzed p62 protein by Western blotting in extracts of HeLa S3 cells that have either been mock infected or infected with HSV-1, using α–p62, an antibody which specifically recognizes p62 (generously provided by Dr. Erich Nigg). The results from these experiments were inconclusive because the intensities of the signals from these Western blots were very weak (data not shown). Other experiments conducted by Alison Kilvert (1995, Unpublished data), using more concentrated protein lysates showed that the abundance of p62 protein after infection does not change in Vero, HeLa S3, and HEL 299 cells.

Indirect immunofluorescence experiments performed by Alison Kilvert (1995, Unpublished data) examined the distribution of p62 in HSV-1 infected cells at 5 hr post-infection. Staining with the α–p62 antibody was generally weak. In mock infected cells some signal was detected faintly in the cytoplasm with brighter staining around the outside of the nucleus, suggesting that p62 is nonnuclear. After infection with KOS1.1, staining of p62 is present throughout the nucleus and around the periphery of the nucleus. The p62 antibody does not appear to cross-react with any other proteins on a Western blot (Kilvert, 1995, Unpublished data). We suspect that the p62 staining does not accurately represent p62 localization. It is possible that the α–p62 antibody does not react specifically in immunofluorescence procedures. Because MO15 is localized to DNA replication compartments after infection (Kilvert, 1995, Unpublished data), this suggests that p62 may also be recruited to viral compartments as both are part of the TFIIH complex. This is assuming that the TFIIH complex remains intact after infection.

Although no detectable alterations are detected in MAT and p62, MO15 protein abundance declines slightly after HSV-1 infection. Immunofluorescence experiments demonstrate that after infection, MO15, and possibly MAT1, are recruited into DNA replication compartments.

G. CDK8

The cyclin C/CDK8 complex is found in the RNAP II holoenzyme and contains a potent CTD kinase activity that remains constant throughout the cell cycle. CDK8 and cyclin C are human homologues of the yeast holoenzyme components SRB10 and SRB11 respectively (Tassan et al., 1995). Cyclin C/CDK8 protein levels do not change during the cell cycle (Rickert et al., 1996). If CDK8 behaves as other members of the CDK family, we would it expect that phosphorylation is required for the protein's activity.

Alison Kilvert (1995, Unpublished data) examined the fate of CDK8 after HSV-1 infection. The protein levels and phosphorylation state of CDK8 did not appear to change after infection. Attempts to examine the distribution of CDK8 by indirect immunofluorescence after infection were unsuccessful. No specific CDK8 staining occurred in mock infected cells using the K35 polyclonal antibody, which is directed against CDK8 (generously provided by Dr. Erich Nigg). High levels of staining occurred in the nucleus and cytoplasm in both mock infected and KOS1.1 infected cells. The K35 antibody may be cross-reacting with some protein other than CDK8.

H. Summary

After HSV-1 infection, TBP (TFIID) (this chapter) and MO15 (TFIIH) (Kilvert, 1995, Unpublished data), specifically localize to DNA replication compartments, along with RNAP II (Chapter 3). Immunofluorescence assays of TFIIB, p56, RAP74, and MAT1 localization are difficult to interpret due to high background signals; however, there appears to be a preferential recruitment of p56, RAP74 and MAT1 to viral replication compartments (Kilvert, 1995, Unpublished data).

TFIIB protein levels decline following HSV-1 infection. MO15 protein levels also decline slightly early after HSV-1 infection. From our Western blots, the protein levels and phosphorylation states of other GTFs, TBP (TFIID), p56 (TFIIE), and RAP74 (TFIIF) appear to be constant after infection with HSV-1. Likewise, the protein levels p62 (TFIIH) and MAT1 (Kilvert, 1995, Unpublished data) do not appear to be altered after infection. Our data suggest that HSV-1 infection does not modify any of these GTFs. In addition, the putative CTD protein kinase CDK8 appears equally abundant before and after infection (Kilvert, 1995, Unpublished data).

It is important to note that we have not examined the activities of the GTFs and the CTD kinases after HSV-1 infection. Although the presence or absence of alterations in GTFs and CTD kinases observed in Western blots and immunofluorescence assays may reflect changes in their respective functions, it is not possible to determine whether the virus targets these GTFs or CTD kinases to preferentially transcribe viral genes. The changes in activity of a particular GTF or CTD kinase, induced by HSV-1 infection, may provide further information on how viral transcription is regulated.

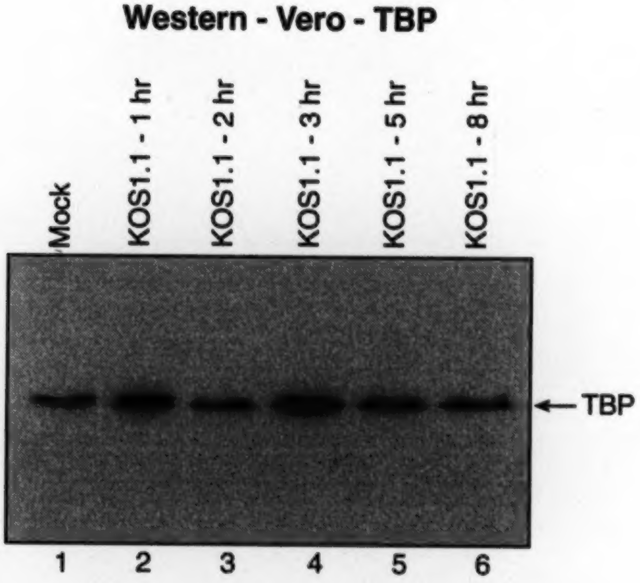
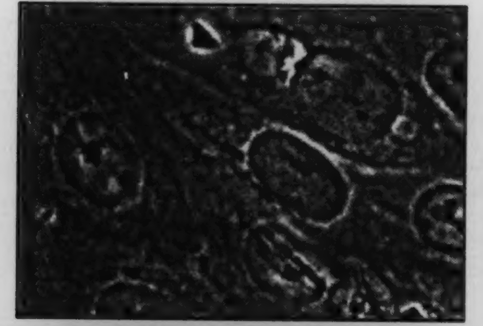

Western - Vero - TBP
Mock
KOS1.1 - 1 hr
KOS1.1 - 2 hr
KOS1.1 - 3 hr
KOS1.1 - 5 hr
KOS1.1 - 8 hr
TBP
1
2
3
4
5
6

Figure 6.1. Western blot analysis of TBP in HSV-1 infected Vero cells. Vero cells were mock infected (lane 1) or infected with HSV-1 (KOS1.1) (lanes 2 to 6) for the times indicated. Cells were harvested and lysed into Laemmli buffer. Extracts were separated by SDS-PAGE (12% acrylamide) and subjected to immunoblotting. The samples were probed with the monoclonal antibody TBP18, which recognizes TBP. TBP migrates at approximately 38 kDa.

Figure 6.2. TBP and ICP8 colocalize in HSV-1 infected Vero cells. Vero cells were infected with HSV-1 (KOS1.1) for 5 hr, fixed and stained with the TBP antibody and anti-ICP8 polyclonal antiserum 3-83. Secondary antibodies were a mixture of rhodamine-conjugated goat anti-mouse immunoglobulin (for TBP visualization) and fluorescein conjugated donkey anti-rabbit immunoglobulin (for ICP8 visualization). Phase-contrast microscopy (panel A). Detection of RNAP II (panel B). Detection of ICP8 (panel C).

Double Immunfluorescence

(A) KOS1.1 - phase



(B) KOS1.1 - TBP

(C) KOS1.1 - ICP8

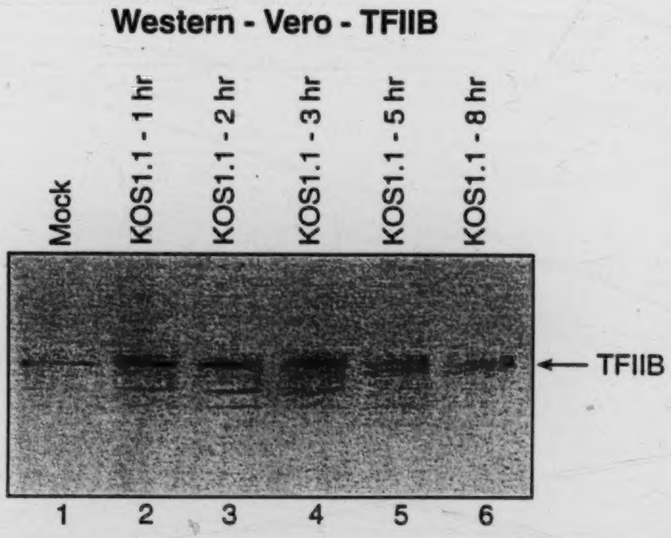

Western - Vero - TFIIB
Mock
KOS1.1 - 1 hr
KOS1.1 - 2 hr
KOS1.1 - 3 hr
KOS1.1 - 5 hr
KOS1.1 - 8 hr
TFIIB
1
2
3
4
5
6

Figure 6.3. Western blot analysis of TFIIB in Vero cells infected with HSV-1. The immunoblot from Fig. 6.1 containing samples of mock infected (lane 1) or HSV-1 (KOS1.1) (lanes 2 to 6) infected Vero cells was stripped as described in Chapter 2 and reprobed with affinity purified polyclonal antibody α-TFIIB antibody, which recognizes TFIIB. TFIIB migrates at approximately 35 kDa.

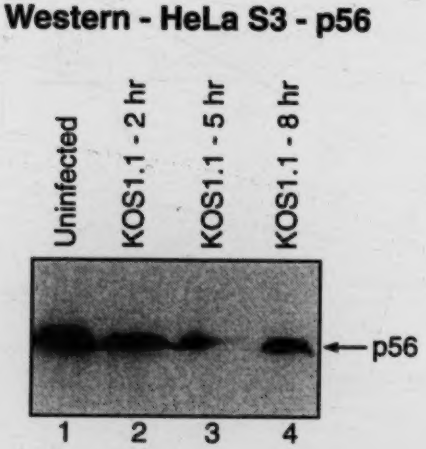

Western - HeLa S3 - p56
Uninfected
KOS1.1 - 2 hr
KOS1.1 - 5 hr
KOS1.1 - 8 hr
p56
1
2
3
4

Figure 6.4. Western blot analysis of the p56 subunit of TFIIE in HeLa S3 cells infected with HSV-1. HeLa S3 cells were uninfected (lane 1) or infected with HSV-1 (KOS1.1) (lanes 2 to 4) for the times indicated. Western blotting was performed as described in the legend to Fig. 6.1. The immunoblot was probed with affinity purified polyclonal antibody α-p56E, which recognizes the p56 subunit of human TFIIE. The p56 subunit migrates at approximately 65 kDa.

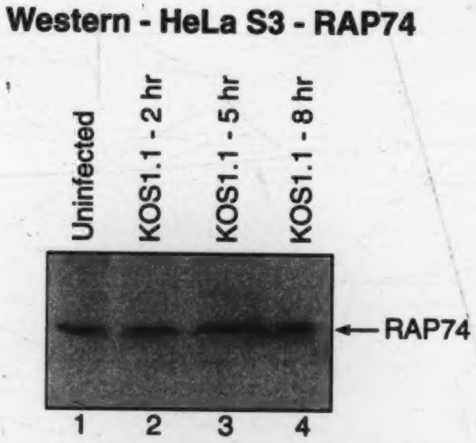

Western - HeLa S3 - RAP74
Uninfected
KOS1.1 - 2 hr
KOS1.1 - 5 hr
KOS1.1 - 8 hr
RAP74
1
2
3
4

Figure 6.5. Western blot analysis of the RAP74 subunit of TFIIF in HeLa S3 cells infected with HSV-1. The immunoblot from Fig. 6.4 containing samples of uninfected (lane 1) and HSV-1 (KOS1.1) (lanes 2 to 4) infected HeLa S3 cells was stripped as described in Chapter 2 and reprobed with affinity purified polyclonal antibody α-RAP74, which recognizes the RAP74 subunit of human TFIIF. The RAP74 subunit migrates at approximately 78 kDa.

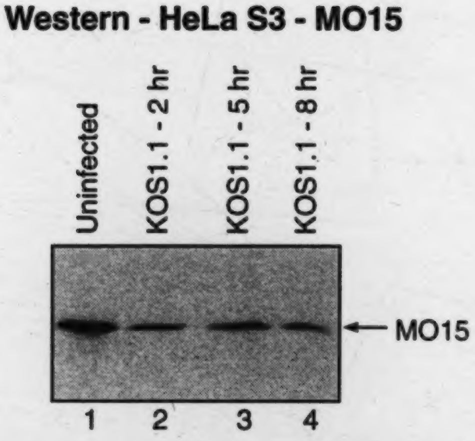

Western - HeLa S3 - MO15
Uninfected
KOS1.1 - 2 hr
KOS1.1 - 5 hr
KOS1.1 - 8 hr
MO15
1
2
3
4

Figure 6.6. Western blot analysis of the MO15 subunit of TFIIH in HeLa S3 cells infected with HSV-1. The immunoblot from Fig. 6.4 and 6.5 containing samples of uninfected (lane 1) and HSV-1 (KOS1.1) (lanes 2 to 4) infected HeLa S3 cells was stripped as described in Chapter 2 and probed with monoclonal antibody MO1.1, which recognizes the MO15 subunit of human TFIIH (Tassan et al., 1994). MO15 migrates at approximately 40 kDa.

CHAPTER SEVEN. ATTENUATION OF DNA-DEPENDENT PROTEIN KINASE ACTIVITY AND ITS CATALYTIC SUBUNIT BY THE HSV-1 TRANSACTIVATOR ICP0 [4]

A. Introduction

HSV-1 infection results in rapid aberrant phosphorylation of the large subunit of RNAP II (Chapter 3). One possible mechanism to explain altered CTD phosphorylation is that virus infection modifies the activity of one or more CTD kinases, resulting in the loss of IIo and the appearance of the intermediately migrating IIi.

CTD kinases are defined as kinases that phosphorylate the consensus heptapeptide repeat of the CTD and cause the characteristic mobility shift of IIa to IIo on SDS-PAGE gels (Dahmus, 1994; Kang and Dahmus, 1995). Putative CTD kinases have been identified *in vitro* but the functions these kinases play *in vivo* are unclear. Mammalian putative CTD kinases include, TFIIH (Lu et al., 1992; Serizawa et al., 1995; Shiekhattar et al., 1995), DNA-dependent protein kinase (DNA-PK) (Arias et al., 1989; Anderson and Lees-Miller, 1992; Dvir et al., 1992; Anderson et al., 1995), the cell cycle control protein p34^{cdc2} (Cisek and Corden, 1989; Zawel et al., 1993), the protein encoded by the *c-abl* proto-oncogene (Baskaran et al., 1996), Tat-associated kinase (TAK) (Herrmann and Rice, 1993; Herrmann and Rice, 1994), CTD-K1 and CTD-K2 (Payne and Dahmus, 1993), and the protein complex of cyclin C and CDK8 (Leclerc et al., 1996; Rickert et al., 1996). The putative CTD kinases are described in Chapter 1.

In the last chapter, I reported the fates of two putative cellular CTD kinases, TFIIH and CDK8, after HSV-1 infection. As described in Chapter 6, there is a modest reduction in the protein abundance of one of the TFIIH subunits, MO15, after infection; however, no alterations are detectable in

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A version of this chapter has been published:

[4] Lees-Miller, S. P., M. C. Long, M. A. Kilvert, V. Lam, S. A. Rice, and C. A. Spencer. 1996. Attenuation of DNA-dependent protein kinase activity and its catalytic subunit by the HSV-1 transactivator ICP0. J. Virol. 70: 7471-7477.

p62, MAT1, or in CDK8. Furthermore, MO15 and MAT1 are relocalized into viral replication compartments after infection, as observed with the large subunit of RNAP II (Chapter 3). The virus may recruit select CTD kinases into DNA replication compartments and alter these kinases in order to induce modifications to RNAP II and perhaps enable efficient viral transcription.

Another putative CTD kinase is the DNA-dependent protein kinase (DNA-PK). DNA-PK (approximately 460 kDa) consists of a catalytic domain (DNA-PKcs, formally known as p350) (Carter et al., 1990; Lees-Miller et al., 1990; Hartley et al., 1995) and a dimeric protein of 70 and 80 kDa, known as Ku, which targets DNA-PK to DNA (Dvir et al., 1992; Gottlieb and Jackson, 1993). DNA-PK plays an important role in DNA double-strand break repair and V(D)J recombination (Anderson, 1994; Jeggo et al., 1995; Roth et al., 1995), and may function in the regulation of RNAP I and RNAP II transcription through its phosphorylation activities (Kuhn et al., 1995; Labhart, 1995; Giffin et al., 1996; Giffin et al., 1997). DNA-PK is a serine/threonine protein kinase that phosphorylates many transcription proteins *in vitro*, including the large subunit of RNAP II, p53, serum response factor, c-Fos, c-Jun, the simian virus 40 large T antigen, and Xenopus TBP (Anderson and Lees-Miller, 1992; Anderson et al., 1995; Labhart, 1996). DNA-PK may repress RNAP I transcription initiation by phosphorylating a component of the RNAP I transcription initiation complex (Kuhn et al., 1995; Labhart, 1995). DNA-PK repression of RNAP II transcription has been observed in glucocorticoid induced transcription from the mouse mammary tumor virus promoter. Repression may be the result of DNA-PK induced phosphorylation of the glucocorticoid receptor and OCT-1 (Giffin et al, 1996; Giffin et al., 1997). In support of DNA-PK's potential role in RNAP II transcription, both Ku and p350/DNA-PKcs are components of a human RNAP II holoenzyme complex (Maldonado et al., 1996).

Because of the potential role of DNA-PK in RNAP II transcription regulation and its ability to phosphorylate the CTD of the large subunit of RNAP II *in vitro*, I was interested in examining the effects of HSV-1 infection on DNA-PK. In this chapter, I report that HSV-1 infection alters the abundance of the p350/DNA-PKcs subunit and DNA-PK activity. The viral IE protein ICP0 is necessary for these effects.

B. DNA-PK's nuclear distribution is not altered after HSV-1 infection

To determine the nuclear distribution of p350/DNA-PKcs during HSV-1 infection, we performed indirect immunofluorescence experiments. HeLa CCL2.2 cells were mock infected or infected with HSV-1 (KOS1.1) for 5 hr. The cells were subsequently fixed and stained with DPK1, a polyclonal antibody that specifically recognizes p350/DNA-PKcs (Lees-Miller et al., 1995; Chan et al., 1996). In mock infected cells, p350/DNA-PKcs displayed strong nuclear staining, with some weaker cytoplasmic staining. The p350/DNA-PKcs subunit was specifically excluded from the nucleoli prior to infection (Fig. 7.1A and 7.1B). After KOS1.1 infection, the distribution of p350/DNA-PKcs was also nuclear; however as nucleoli disintegrate after infection, staining was not excluded from nucleoli. After infection, the majority of p350/DNA-PKcs was dispersed throughout the nucleus, while minor amounts of the protein were present in the cytoplasm (Fig. 7.1D). The cells were successfully infected, as indicated by ICP8 staining and efficient DNA replication compartment formation (data not shown).

We also examined the localization of Ku during HSV-1 infection by indirect immunofluorescence. HeLa CCL2.2 cells were mock infected or infected with KOS1.1 for 5 hr. The cells were fixed and stained with Ku24.2, a mouse polyclonal antibody directed against both p70 and p80 subunits (Chan et al., 1996). In mock infected cells, Ku was localized throughout the nucleus, including the nucleoli (Fig. 7.2A and 7.2B). KOS1.1 infection resulted in no obvious changes to Ku localization (Fig. 7.2D). The cells were efficiently infected with HSV-1 as shown by the presence of ICP8 staining and efficient DNA replication compartment formation (data not shown).

The data demonstrate that after HSV-1 infection, p350/DNA-PK and Ku remain nuclear. Although these proteins are not specifically recruited into DNA replication compartments after infection, neither do they appear to be excluded.

C. DNA-PK activity and the p350/DNA-PKcs subunit decline following HSV-1 infection

To test whether p350/DNA-PKcs protein levels were altered (i.e. phosphorylation state or protein abundance) after HSV-1 infection, I analyzed p350/DNA-PKcs by Western blotting. HeLa S3 cells were mock infected or infected with HSV-1 (KOS1.1) for various times. Cell extracts were prepared (at equal numbers of cells/µl of extract) and analyzed by SDS-PAGE and Western blotting with DPK1, an antibody which specifically recognizes p350/DNA-PKcs (Lees-Miller et al., 1995; Chan et al., 1996) (Fig. 7.3A). Mock infected cells showed constant levels of p350/DNA-PKcs protein (Fig. 7.3A, lanes 1 to 3), whereas KOS1.1 infected cells showed severe depletions in p350/DNA-PKcs protein (Fig. 7.3A, lanes 4 to 6 and Table 7.1), over a 6 hr time period. The most significant reduction occurred between 2 and 4 hr post-infection, where protein levels dropped from 88.2% to 16.4% of mock levels (Table 7.1). After virus infection, there were no detectable changes in the migration of the p350/DNA-PKcs protein, suggesting that its phosphorylation state remained unaltered.

To determine if reductions in Ku p70 and Ku p80, were observed after HSV-1 infection, I analyzed Ku by Western blotting. HeLa S3 cells were mock infected or infected with HSV-1 (KOS1.1). Cells were harvested (at equal numbers of cells/µl of extract) at various post-infection times and total proteins were separated on SDS-PAGE gels. Western blotting was performed with Ku24.2, a polyclonal antibody directed against the p70 and p80 subunits of Ku (Chan et al., 1996) (Fig. 7.3B). Mock infected HeLa S3 cells showed constant levels of Ku p80 from 0 to 6 hr post-infection (Fig. 7.3B, lanes 1 to 3). After KOS1.1 infection, Ku p80 protein levels also remained abundant up to 6 hr post-infection (Fig. 7.3B, lanes 4 to 6). In this particular experiment, the Ku24.2 antibody reacted weakly with the p70 subunit. In other experiments, the reaction of the Ku24.2 antibody was much stronger (Fig. 7.3 and data not shown). After infection, there were no changes in the migration of Ku p70 and p80 subunits, suggesting that their phosphorylation states are not altered.

To determine if the loss of p350/DNA-PKcs protein correlated with a loss in DNA-PK kinase activity, Dr. Susan Lees-Miller, University of Calgary, Calgary, Alberta (1996) performed DNA-PK activity assays on HSV-1 infected HeLa S3 cell extracts. In these experiments, HeLa S3 cells were mock infected or KOS1.1 infected and harvested at various times. The cell extracts were assayed for DNA-PK activity using the PESQEAFADLWKK substrate peptide (Allalunis-Turner et al., 1995; Anderson et al., 1995; Chan et al., 1996). DNA-PK activity and p350/DNA-PKcs protein abundance declined in parallel in KOS1.1 infected cell extracts (Table 7.1). DNA-PK activity was approximately 14% of mock levels and p350/DNA-PKcs protein levels were approximately 9% of mock infected cells at 6 hr post-infection.

We then determined whether the depletion of p350/DNA-PKcs protein observed after infection was specific to HeLa S3 cells or if this also occurred in another cell line. Western blotting experiments using the p350/DNA-PKcs antibody, DPK1, were performed on extracts from HEL 299 and HeLa CCL2.2 cells infected with HSV-1 (data not shown). After infection, p350/DNA-PKcs protein levels rapidly decreased in HEL 299 and HeLa CCL2.2 cells, with similar kinetics as observed in HeLa S3 cells. These results demonstrate that p350/DNA-PKcs is altered after HSV-1 infection in at least three different human cell lines. Furthermore, experiments performed by Kilvert (1995, Unpublished data), showed declines in p350/DNA-PKcs abundance after HSV-2 infection in HeLa S3 cells.

These data demonstrate that after HSV-1 infection in HeLa S3, HEL 299, and HeLa CCL2.2 cells, p350/DNA-PKcs protein levels are altered, as detected by Western blotting. Furthermore, the reduction in protein abundance parallels a reduction in DNA-PK activity.

D. The half-life of p350/DNA-PKcs decreases following HSV-1 infection

It is possible that reductions in p350/DNA-PKcs following HSV-1 infection are the result of virus-induced shutoff of host cell translation (Smiley et al., 1991). The shutoff of host cell translation may cause a general depletion of all labile cellular proteins. If the translation of p350/DNA-PKcs is suppressed and if the half-life of p350/DNA-PKcs is short, we would expect to see a loss of p350/DNA-PKcs protein. However, in human erythroleukemia cells, the half-life of p350/DNA-PKcs is reported to be greater than 5 days (Ajmani et al., 1995). In addition, the half-life of p350/DNA-PKcs is greater than 24 hr in mock infected cells but less than 4 hr in KOS1.1 infected cells (Lees-Miller et al., 1996). This rapid depletion of p350/DNA-PKcs observed early (within 3 to 4 hr) in virus infection suggests that an active p350/DNA-PKcs degradation process is induced in HSV-1 infected cells.

To test whether the degradation of cellular proteins is a general effect of HSV-1 infection, we examined the abundance of two other cellular proteins, protein kinase C (PKC) (Fig. 7.4) and phosphatase-1 (Fig. 7.5), following infection. HeLa S3 cells were uninfected or infected with HSV-1 (KOS1.1), and total proteins were harvested and separated on SDS-PAGE gels. Western blotting was performed using either an antibody recognizing the C-terminus of PKC or an antibody recognizing the C-terminus (amino acids 316 to 330) of phosphatase-1. As shown in Fig. 7.4, PKC protein levels appeared to vary after KOS1.1 infection. PKC protein migrated at approximately 75 kDa and decreased after 1 hr post infection and then steadily increased up to 8 hr post-infection. Between 8 and 12 hr post-infection, there was a decrease in PKC abundance (Fig. 7.4, lanes 7 and 8). Slight variations in phosphatase-1 protein levels were also observed after KOS1.1 infection (Fig. 7.5). Phosphatase-1 migrated at approximately 37 kDa. At 1 hr post-infection, there was an increase in the abundance of phosphatase-1, as compared to uninfected cells (Fig. 7.5, lanes 2 and 3). Phosphatase-1 protein levels decreased at 2 hr but then increased at 3 hr post-infection (Fig. 7.5, lanes 4 and 5). After 3 hr and up to 12 hr post-infection, the phosphatase-1 protein levels stabilized to near constant levels (Fig. 7.5, lanes 5 to 8). These data demonstrate that although minor variations may occur in the levels of PKC and phosphatase-1 protein, there are no severe reductions in protein abundance after HSV-1 infection. The fluctuations in protein levels of PKC and phosphatase-1 are not likely due to unequal loading of sample in each lane, because the same immunoblot was used to probe for PKC or phosphatase-1 (Fig. 7.4 and 7.5). The changes in PKC protein are not similar to the changes in phosphatase-1 protein after infection. In addition, we have previously found that the abundance of TBP, p56 (TFIIE), RAP74 (TFIIF), and most other members of the RNAP II transcription machinery do not change significantly up to 8 hr post-infection (Chapter 6). Therefore, the depletion of p350/DNA-PK after infection appears not to be part of a general decline in cellular protein levels.

These data indicate that the rapid elimination of p350/DNA-PKcs is due to a shorter half-life in HSV-1 infected cells, as compared to mock infected cells. Furthermore, these results suggest that an active p350/DNA-PKcs degradation process is induced in infected cells, since there does not appear to be a general degradation of all cellular proteins. The mechanism by which p350/DNA-PK protein is depleted after infection is unknown. No specific breakdown products were observed on the immunoblots, suggesting that the degradation of p350/DNA-PKcs occurs rapidly and results in small fragments following infection (Fig. 7.3, lanes 4 to 6 and data not shown).

E. Depletion of DNA-PK activity and p350/DNA-PKcs protein require expression of the viral IE genes

One explanation for the reduction in p350/DNA-PKcs abundance is that a component of the virion could alter the protein's half-life. The HSV-1 virion contains several regulatory proteins, including VP16 which is required for transcription activation of the viral IE genes, and the *vhs*-encoded protein which is instrumental in shutoff of host cell translation and the decline in many host RNAs (Smiley et al., 1991). To determine if the depletion of DNA-PK activity and p350/DNA-PKcs protein levels are the consequence of the functions of the virion components, we examined DNA-PK activity and p350/DNA-PKcs protein abundance in cells infected with UV-inactivated virus. The stock of UV-inactivated virus has been tested previously and contains active virion proteins capable of transactivating an IE promoter (Chapter 3). Shortwave UV treated HSV-1 virus crosslinks viral DNA and prevents transcription of all viral genes but leaves the virion proteins intact and active (Rice et al., 1994).

HeLa S3 cells were mock infected or infected with KOS1.1 or UV-inactivated virus. Cells were harvested and analyzed for p350/DNA-PKcs or Ku abundance by Western blotting. The Western blots shown in Fig 7.6 and the activity assays shown in Fig. 7.7 were provided by Dr. Susan Lees-Miller (1996). Infection with UV-inactivated virus resulted in no changes in the abundance of p350/DNA-PKcs or Ku p70 or Ku p80 (Fig. 7.6A, lanes 4 and 5). DNA-PK activity also remained constant for up to 8 hr after infection with UV-inactivated virus (Fig. 7.7A, line with open squares). These data demonstrate that the expression of the viral genome is necessary for the loss of both p350/DNA-PKcs and DNA-PK activity. Virion components alone are not sufficient for the depletion of p350/DNA-PKcs or kinase activity.

To test whether viral DNA replication, DNA packaging, or the expression of DE and L gene products are necessary for the depletion of p350/DNA-PKcs protein and DNA-PK activity, we analyzed protein levels and kinase activity in cells infected with a virus containing a deletion in the ICP4 gene. The expression of ICP4 is absolutely essential for viral DE and L gene expression and for viral DNA replication and packaging (Preston, 1979; Watson and Clements, 1980; DeLuca et al., 1984; DeLuca and Schaffer, 1985; Godowski and Knipe, 1986). HeLa S3 cells were mock infected or infected with *d*120, an ICP4 deletion mutant (DeLuca et al., 1985). Cells were harvested and analyzed for p350/DNA-PKcs or Ku by Western blotting. In ICP4 mutant infected cells, there was a decrease in p350/DNA-PKcs protein levels (Fig. 7.6B), similar to that observed in KOS1.1 infected cells (Fig. 7.6A, lanes 2 and 3 and Fig. 7.6A, lanes 4 to 6). The levels of Ku p70 and Ku p80 remained unchanged up to 8 hr post-infection with *d*120. DNA-PK activity in these extracts was also reduced (Fig. 7.7A, line with crosses) and paralleled the loss of p350/DNA-PKcs protein. These results indicate that ICP4, DE and L gene expression, viral DNA replication, and DNA packaging are not required for the depletion of p350/DNA-PKcs protein and kinase activity. Therefore, the expression of another viral IE protein other than ICP4 is necessary to induce the reductions in p350/DNA-PKcs protein and DNA-PK activity.

F. The ICP0 gene product is required for modifications to DNA-PK and its catalytic subunit

To identify which viral IE proteins are required for the loss of p350/DNA-PKcs protein and DNA-PK activity after HSV-1 infection, HeLa S3 cells were infected with viruses mutant in each of the IE genes. HeLa S3 cells were infected with virus mutant in the IE genes encoding ICP22 (*n*199), ICP27 (*d*27-1), ICP0 (*n*212), and ICP6 (ICP6). Each of the mutant viruses have been described in Chapter 2 and 4. Cells were harvested and analyzed for p350/DNA-PKcs or Ku abundance by Western blotting. Protein levels of p350/DNA-PKcs were depleted in cells infected with viruses mutant in the genes encoding ICP22 (Fig. 7.6C), ICP27 (Fig. 7.6D), and ICP6 (Fig. 7.6E), with similar kinetics as observed in cells infected with WT HSV-1 (Fig. 7.3A, lanes 4 to 6 and Fig. 7.6, lanes 2 and 3). However, cells infected with a virus mutant in the gene encoding ICP0, *n*212, retained constant levels of p350/DNA-PKcs, as in mock infected cells (Fig. 7.6F, top panel). We confirmed that the ICP0 mutant virus infection occurred efficiently by probing the same Western blot for the expression of the ICP4 protein (Fig. 7.6F, middle panel). Ku p70 and Ku p80 protein levels remained constant in all infections (Fig. 7.6A to 7.6F). These data indicate that ICP0 is required for the efficient depletion of p350/DNA-PKcs protein levels.

We have also probed the Western blot shown in Fig. 7.3A for the expression of the ICP0 protein (Fig. 7.8). The Western blot was stripped and reprobed with H1112, a mouse monoclonal antibody which recognizes ICP0. ICP0 protein levels increased (Fig 7.8, lanes 4 to 6) as p350 protein levels decreased (Fig 7.3A, lanes 4 to 6) following HSV-1 (KOS1.1) infection. At 2 hr post-infection, p350/DNA-PKcs protein levels declined slightly to 88.2% of mock infected cells (Table 7.1), while only small amounts of ICP0 were expressed (Fig 7.8, lane 4). At 4 and 6 hr post-infection, p350/DNA-PKcs protein levels dropped significantly to below 16.4% of mock levels (Table 7.1) and large amounts of ICP0 were expressed (Fig. 7.8, lane 6). This data supports what we have previously shown, that the depletion of p350/DNA-PKcs protein occurs in the presence of ICP0.

DNA-PK activity was assayed in the mutant infected extracts. Infection of the cells with viruses mutant in ICP22, ICP27, or ICP6 resulted in a loss of DNA-PK activity, similar to that seen after infection with WT HSV-1 (Fig. 7.7A, lines with closed triangles, open triangles, and diamonds respectively). The decline in DNA-PK activity corresponded to the loss in p350/DNA-PK protein. Infection of the cells with the ICP0 mutant virus, *n*212, resulted in high and constant DNA-PK activity, similar to mock infected cells (Fig. 7.7A, line with open circles versus line with filled squares). These data indicate that ICP0 is required for the efficient depletion of DNA-PK activity.

In all infections, Ku p70 and Ku p80 protein levels were found to be constant up to 8 hr. To test whether Ku was functional when DNA-PK activities were low, add-back experiments were performed by Dr. Susan Lees-Miller (1996). Extracts from cells infected with WT virus or viruses mutant in the IE gene encoding ICP4, ICP6, or ICP27 were reconstituted with purified p350/DNA-PKcs and assayed for DNA-PK activity (Fig. 7.7B). Purified p350/DNA-PKcs restored DNA-PK activity in all extracts depleted of activity after infection. This demonstrates that Ku remained functional after HSV-1 infection.

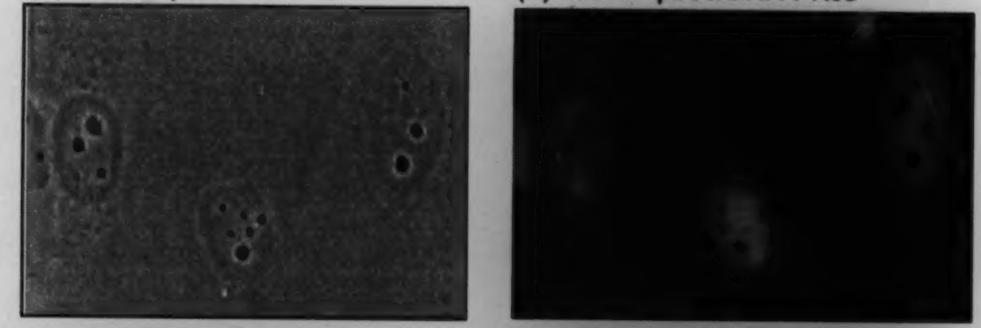
G. Summary

The nuclear distribution of DNA-PK remains unaltered after HSV-1 infection. Both p350/DNA-PKcs and Ku are not specifically recruited to, nor do they appear to be excluded from, DNA replication compartments following infection.

While other cellular proteins, such as PKC, phosphatase-1, and Ku (p70 and p80) remain abundant, the p350/DNA-PKcs subunit is severely depleted after HSV-1 infection. The half-life of p350/DNA-PKcs is reduced to less than 4 hr after infection, whereas the half-life of the protein is over 24 hr in mock infected cells (Lees-Miller et al., 1996). This depletion in p350/DNA-PKcs protein levels parallels declines in DNA-PK activity (Lees-Miller et al., 1996). We conclude that infection of human cells with HSV-1 specifically targets the p350/DNA-PKcs for degradation and attenuates DNA-PK kinase activity, while maintaining the DNA binding function of

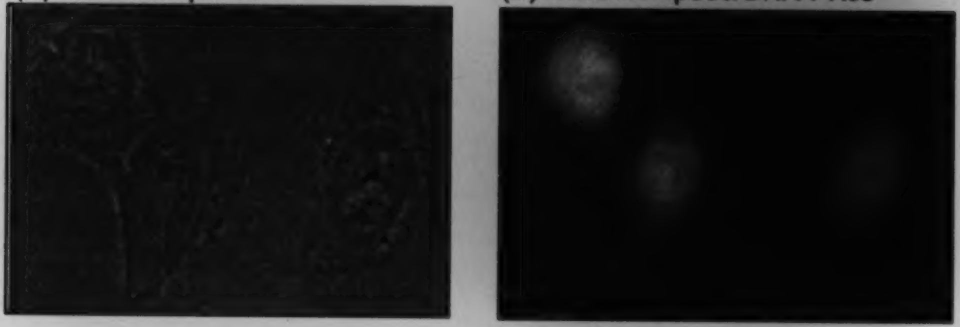
Ku. Furthermore, the depletion of p350/DNA-PKcs protein and DNA-PK kinase activity after HSV-1 infection is dependent on ICP0.

Immunofluorescence - p350/DNA-PKcs



(A) Mock - phase

(B) Mock - p350/DNA-PKcs



(C) KOS1.1 - phase

(D) KOS1.1 - p350/DNA-PKcs

Figure 7.1. Nuclear localization of p350/DNA-PKcs. HeLa CCL2.2 cells were mock infected (panels A and B) or infected with HSV-1 (KOS1.1) (panels C and D) for 5 hr. The cells were fixed and stained with the polyclonal antibody DPK1, that recognizes p350/DNA-PKcs. Secondary antibodies were α-rabbit biotinylated antibody and α-streptavidin fluorescein. Phase-contrast microscopy (panels A and C). Fluorescence microscopy (panels B and D).

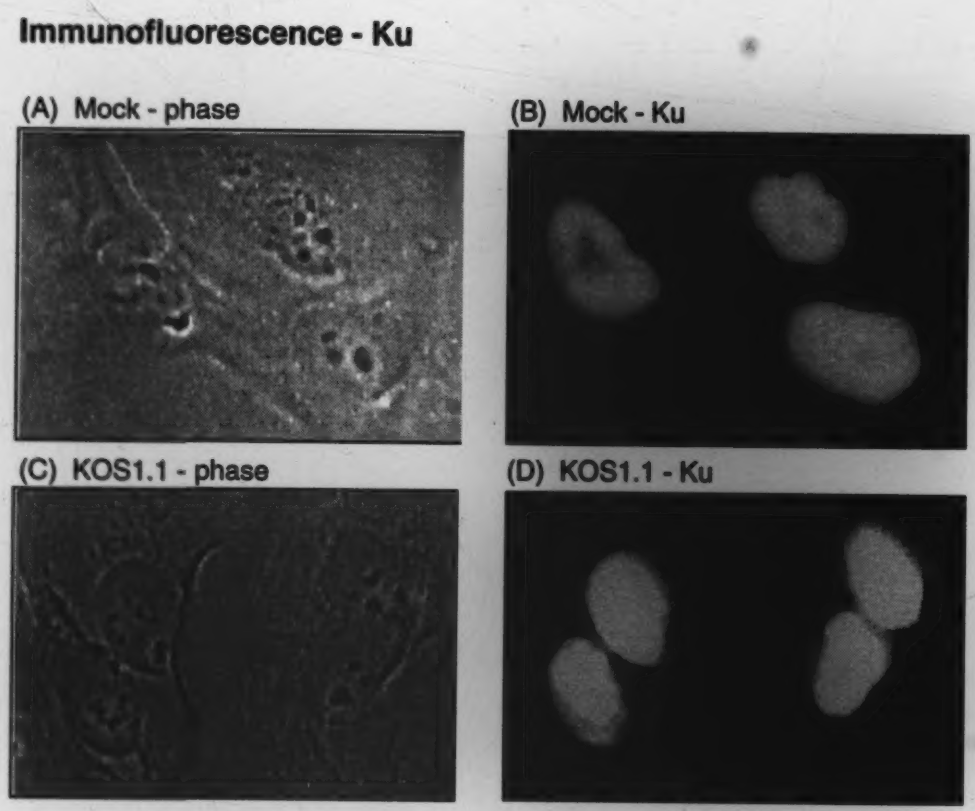

Immunofluorescence - Ku
(A) Mock - phase
(B) Mock - Ku
(C) KOS1.1 - phase
(D) KOS1.1 - Ku

Figure 7.2. Nuclear localization of Ku. HeLa CCL2.2 cells were mock infected (panels A and B) or infected with HSV-1 (KOS1.1) (panels C and D) for 5 hr. The cells were fixed and stained with the polyclonal antibody Ku24.2, that recognizes both Ku p70 and Ku p80. Secondary antibodies were α-mouse biotinylated antibody and α-streptavidin Texas Red. Phase-contrast microscopy (panels A and C). Fluorescence microscopy (panels B and D).

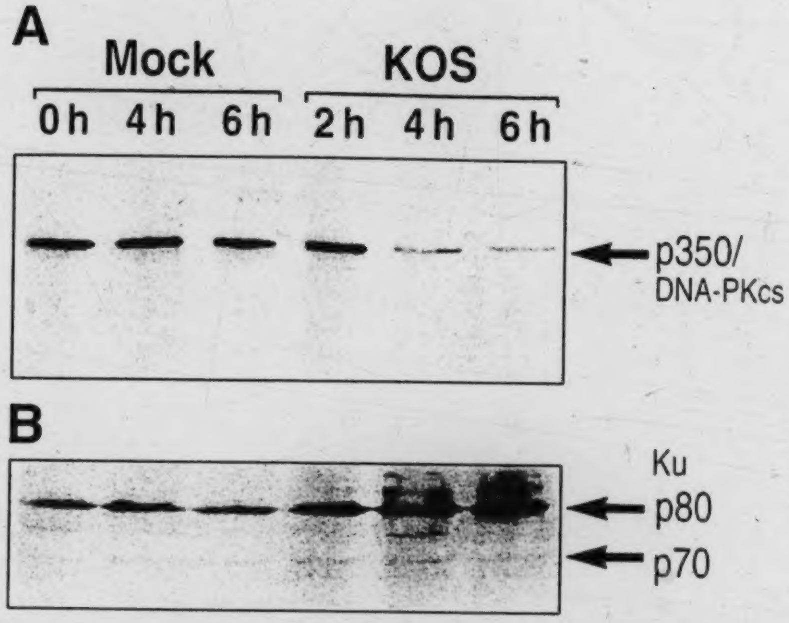


Figure 7.3. Depletion of p350/DNA-PKcs protein in HeLa S3 cells following HSV-1 (KOS1.1) infection. (A) Western blot of p350/DNA-PKcs. HeLa S3 cells were mock infected (lanes 1 to 3) or infected with HSV-1 (KOS1.1) (lanes 4 to 6). Cells were harvested at the times indicated and lysed into Laemmli buffer. Total proteins were separated by SDS-PAGE (8% acrylamide) and subjected to immunoblotting. The samples were analyzed for p350/DNA-PKcs using the polyclonal antibody DPK1. (B) Western blot of Ku p70 and Ku p80. The same samples analyzed in panel A were analyzed for Ku p70 and Ku p80 subunits by using Ku polyclonal antibody Ku24.2. The Ku p70 cross-reacted weakly on this particular blot but was visible on longer exposures and in other experiments analyzing mock infected and KOS1.1 infected cells (see Fig. 7.6).

TABLE 7.1. Depletion of DNA-PK activity and p350/DNA-PKcs protein following HSV-1 infection

	DNA-PK activity[a]		p350/DNA-PKcs level	
Sample	U/mg protein	% of mock, 6 hr	Integral[b]	% of mock, 6 hr
Mock				
0 hr	8.26			
4 hr	9.88			
6 hr	9.09	100	2,949	100
KOS				
2 hr	7.28	80.1	2,601	88.2
4 hr	2.27	24.9	485	16.4
6 hr	1.27	13.9	261	8.9

[a] Activity in extracts prepared from the same set of samples as analyzed in Fig. 7.3. Each value is the average of duplicate assays. DNA-PK activity assays were performed by Dr. Susan Lees-Miller (1996).

[b] Integral under peak determined by densitometer scans of autoradiograph shown in Fig. 7.3A. Densitometry analysis was performed by Alison Kilvert (Lees-Miller et al., 1996).

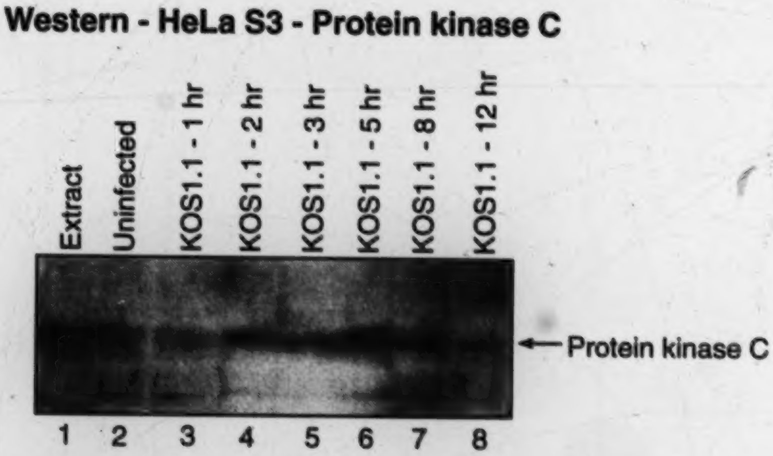

Western - HeLa S3 - Protein kinase C
Extract
Uninfected
KOS1.1 - 1 hr
KOS1.1 - 2 hr
KOS1.1 - 3 hr
KOS1.1 - 5 hr
KOS1.1 - 8 hr
KOS1.1 - 12 hr
Protein kinase C
1
2
3
4
5
6
7
8

Figure 7.4. Western blot analysis of protein kinase C in HeLa S3 cells infected with HSV-1. HeLa S3 cells were uninfected (lane 2) or infected with HSV-1 (KOS1.1) (lanes 3 to 8) for the times indicated. Cells were harvested and lysed into Laemmli buffer. Extracts were separated by SDS-PAGE (12% acrylamide) and subjected to immunoblotting. Lane 1 contained an aliquot of a nuclear *in vitro* transcription extract prepared from HeLa cells. The samples were probed with a polyclonal antibody that recognizes the C terminus of protein kinase C. Protein kinase C migrates at approximately 75 kDa.

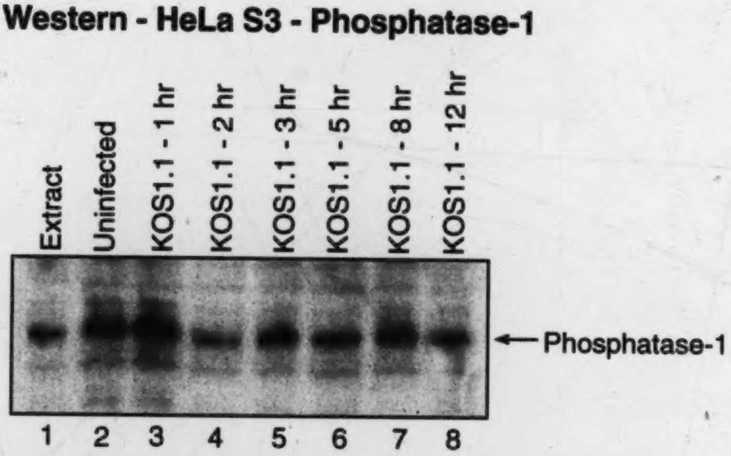
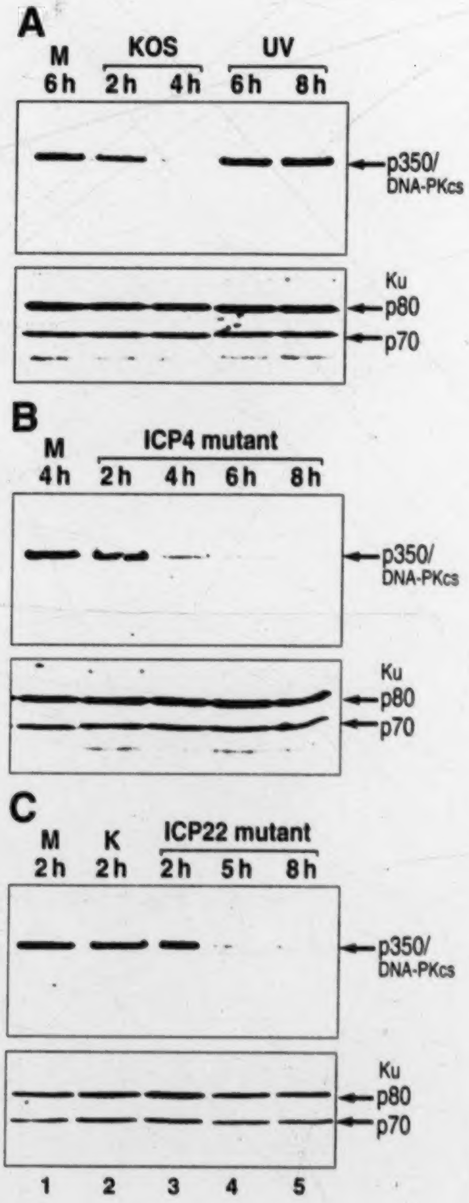

Western - HeLa S3 - Phosphatase-1
Extract
Uninfected
KOS1.1 - 1 hr
KOS1.1 - 2 hr
KOS1.1 - 3 hr
KOS1.1 - 5 hr
KOS1.1 - 8 hr
KOS1.1 - 12 hr
Phosphatase-1
1
2
3
4
5
6
7
8

Figure 7.5. Western blot analysis of phosphatase-1 in HeLa S3 cells infected with HSV-1 (KOS1.1). The immunoblot from Fig. 7.4 was stripped as described in Chapter 2 and probed with a polyclonal antibody that recognizes the C terminus of phosphatase-1. Phosphatase-1 migrates at approximately 37 kDa. Lane 1 contained an aliquot of a HeLa nuclear *in vitro* transcription extract. Lane 2 is a cell extract from uninfected HeLa S3 cells. Lanes 3 to 8 are cells extracts from HeLa S3 cells that have been infected with HSV-1 (KOS1.1) for the times indicated.

Figure 7.6. Requirement for ICP0 in the decline of p350/DNA-PKcs protein levels. HeLa S3 cells were mock infected or infected with WT HSV-1 (KOS1.1), UV-inactivated virus or viruses mutant in IE genes. Cells were harvested, extracts were prepared for DNA-PK assays, and aliquots containing 10 µg of protein were analyzed by immunoblotting and probed with DPK1, Ku24.2, or H1101 antibodies. DPK1, Ku24.2, and H1101 recognize p350/DNA-PKcs, Ku and ICP4 respectively. (A) Viral gene expression is required for the loss of p350/DNA-PKcs protein. Lanes: 1, cells mock infected (M) for 6 hr; 2 and 3, cells infected with KOS1.1 for 2 or 4 h; 4 and 5, cells infected with UV-inactivated virus for 6 and 8 h. (B) An IE gene product, other than ICP4, is required for the loss of p350/DNA-PKcs protein. Lanes: 1, cells mock infected for 4 h; 2 to 5, cells infected with the ICP4 deletion mutant *d*120 for the times indicated. (C) The IE gene product, ICP22, is not required for the loss of p350/DNA-PKcs protein. Lanes: 1, cells mock infected for 2 h; 2, cells infected with KOS1.1 for 2 h; 3 to 5, cells infected with the ICP22 nonsense mutant 22/*n*199 for the times indicated. (D) The IE gene product ICP27 is not required for the loss of p350/DNA-PKcs protein. Lanes: 1, cells mock infected for 2 h; 2 to 5, cells infected with the ICP27 null mutant *d*27-1 for the times indicated. (E) The DE gene product ICP6 is not required for the loss of p350/DNA-PKcs protein. Lanes: 1, cells mock infected for 2 h; 2 to 5, cells infected with the ICP6 deletion mutant ICP6 for the times indicated. (F) The IE gene product ICP0 is required for the loss of p350/DNA-PKcs protein. Lanes: 1, cells mock infected for 2 h; 2 to 5, cells infected with the ICP0 nonsense mutant *n*212. In the middle panel, the same blot that was probed for p350/DNA-PKcs was stripped and reprobed with H1101 against ICP4. (A to F) Ku protein remains constant in mock and virus infected cells. Western blotting was performed by Dr. Susan Lees-Miller (1996).


A
M 6h
KOS
2h 4h
UV
6h 8h
p350/
DNA-PKcs
Ku
p80
p70
B
M 4h
ICP4 mutant
2h 4h 6h 8h
p350/
DNA-PKcs
Ku
p80
p70
C
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Ku
p80
p70
1 2 3 4 5

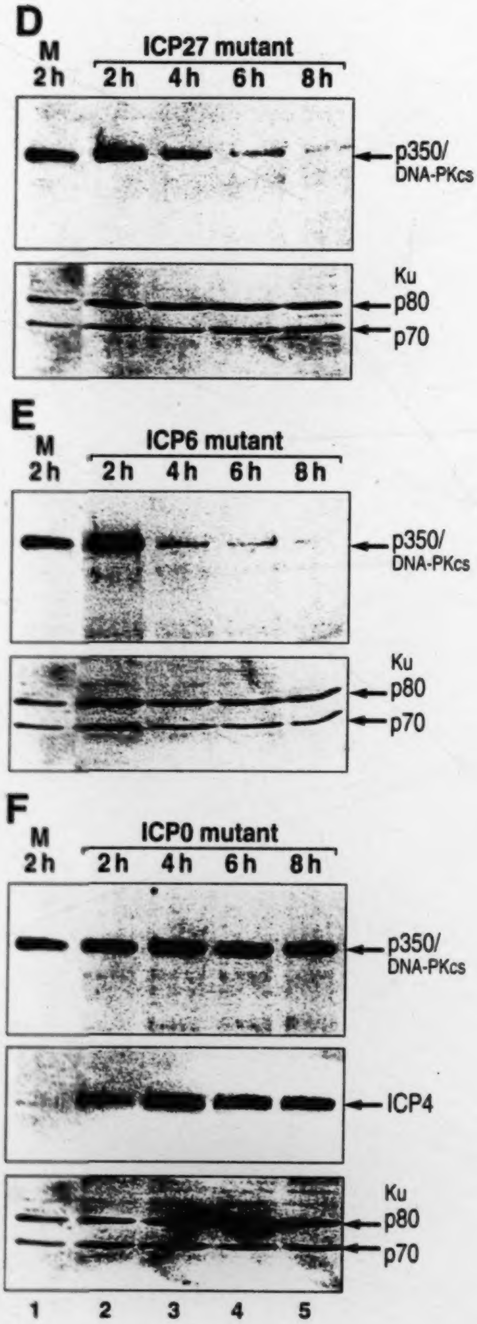
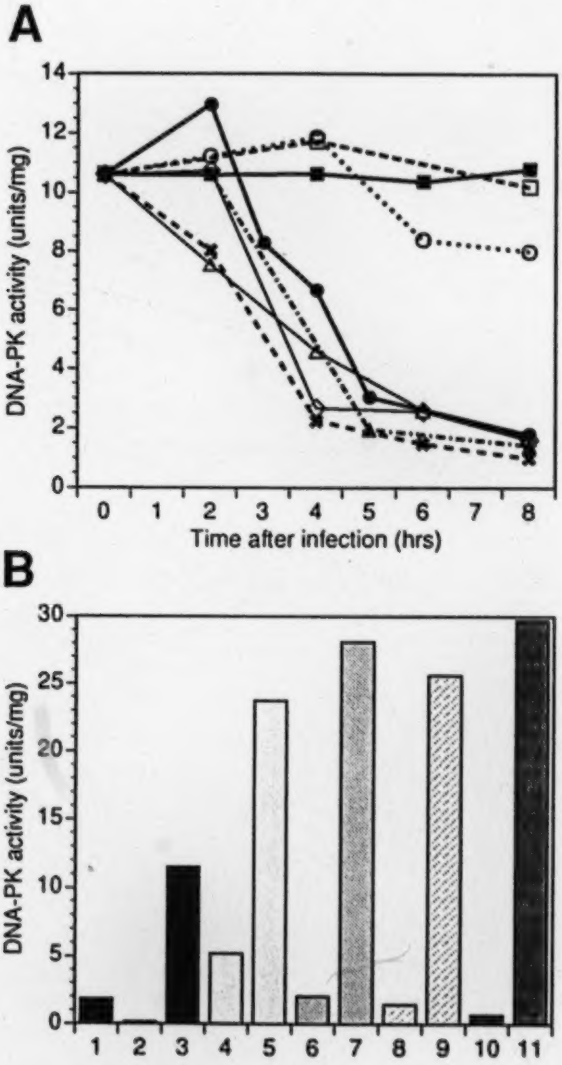

D
M
2h
ICP27 mutant
2h
4h
6h
8h
p350/
DNA-PKcs
Ku
p80
p70
E
M
2h
ICP6 mutant
2h
4h
6h
8h
p350/
DNA-PKcs
Ku
p80
p70
F
M
2h
ICP0 mutant
2h
4h
6h
8h
p350/
DNA-PKcs
ICP4
Ku
p80
p70
1
2
3
4
5

Figure 7.7. Loss of DNA-PK activity and retention of Ku activity following infection with WT HSV-1 (KOS1.1) and mutant viruses. HeLa S3 cells were mock infected or infected with viruses as described in the legend to Fig. 7.6. (A) DNA-PK activity in infected cell extracts. Cells were harvested, extracts were prepared, and DNA-PK activity assays were performed as described in Chapter 2 and Rice et al. (1996). Cells were mock infected (filled squares) or infected with UV-inactivated virus (open squares), KOS1.1 (filled circles), ICP0 mutant *n*212 (open circles), ICP4 mutant *d*120 (crosses), ICP6 mutant ICP6 (diamonds), ICP27 mutant *d*27-1 (open triangles) or ICP22 mutant 22/*n*199 (closed triangles). (B) Add-back experiments. Bars: 1, p350/DNA-PKcs alone; 2, Ku alone; 3, p350/DNA-PKcs plus Ku; 4, KOS1.1 infected extract alone; 5, KOS1.1 infected extract plus p350/DNA-PKcs; 6, ICP4 mutant *d*120 infected extract alone; 7, ICP4 mutant *d*120 infected extract plus p350/DNA-PKcs; 8, ICP6 mutant ICP6 infected extract alone; 9, ICP6 mutant ICP6 infected extract plus p350/DNA-PKcs; 10, ICP27 mutant *d*27-1 infected extract alone; 11, ICP27 mutant *d*27-1 infected extract plus p350/DNA-PKcs. Assays were performed by Dr. Susan Lees-Miller (1996).


A
DNA-PK activity (units/mg)
14
12
10
8
6
4
2
0
0
1
2
3
4
5
6
7
8
Time after infection (hrs)
B
DNA-PK activity (units/mg)
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
11

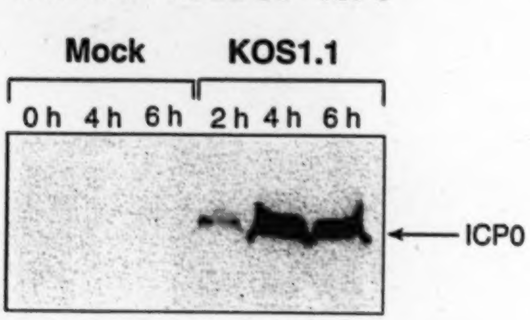

Western - HeLa S3 - ICP0
Mock
KOS1.1
0 h
4 h
6 h
2 h
4 h
6 h
ICP0

Figure 7.8. Western blot analysis of ICP0 in WT HSV-1 infected HeLa S3 cells. The immunoblot of Fig. 7.3A containing samples of mock infected (lanes 1 to 3) or WT HSV-1 (KOS1.1) infected HeLa S3 cells (lanes 4 to 6) was stripped as described in Chapter 2 and reprobed with H1112, a mouse monoclonal antibody that recognizes ICP0. ICP0 migrates at approximately 110 kDa.

CHAPTER EIGHT. THE ANALYSIS OF THE LARGE SUBUNIT OF RNAP II AFTER INFECTION WITH HUMAN DNA VIRUSES

A. Introduction

The Herpesviridae family is divided into three subfamilies, α-, β-, and γ-herpesvirinae (Table 1.1). Human α-herpesviruses include herpes simplex type 1 (HSV-1), herpes simplex type 2 (HSV-2), and varicella-zoster virus (VZV). Human β- and γ-herpesviruses include human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) respectively. All herpesviruses are enveloped, contain an icosahedral nucleocapsid, and are 150 to 200 nm in diameter. The genomes of the viruses consist of one molecule of double-stranded DNA of approximately 120 to 240 kb. Transcription and replication are coordinately regulated and sequentially ordered in a cascade fashion (Murphy and Kingsbury, 1990).

In the studies reported in this chapter, I sought to determine whether the modifications to RNAP II that we observe after HSV-1 infection also occur after infection with other human DNA viruses. I examined the large subunit of RNAP II after infection with viruses that belong to the herpesvirus (α and β) and poxvirus family. We chose to study herpesviruses which are closely related to HSV-1 (HSV-2 and VZV), more distantly related herpesvirus (HCMV and HHV-6), and a cytoplasmic DNA virus, vaccinia virus (VV).

B. Herpes simplex virus type 2 (HSV-2) infection results in RNAP II modifications

HSV-2, an α-herpesvirus, shares many commonalties with HSV-1, in terms of morphology, DNA replication, and gene transcription. Although HSV-1 and HSV-2 are usually transmitted by different routes and involve different areas of the body, the two viruses have extensive DNA and protein sequence identity (Whitley, 1990). The HSV-1 and HSV-2 genomes contain similar G+C contents, 67% and 69% respectively (Roizman et al., 1991). The predicted amino acid sequences for ICP8 and DNA polymerase of HSV-1 and HSV-2 reveal a 97.2% and 95.5% identity respectively (Tsurumi et al., 1987; Toh et al., 1993). In addition, thymidine kinase, glycoprotein C, alkaline exonuclease, and ribonucleotide reductase amino acid sequences are respectively, 86%, 82%, 91% and 93% identical in HSV-1 and HSV-2 (Draper et al., 1986).

The regulation of transcription is similar in both HSV-1 and HSV-2. Cellular gene transcription is suppressed while viral transcription is activated after infection. Both viruses transcribe their genome in the nucleus and are dependent on the host's RNAP II transcription machinery for transcription of viral genes. Viral gene transcription occurs as a temporal cascade and is regulated primarily at the transcriptional level (Roizman and Sears, 1990). Based on the similarities between HSV-1 and HSV-2, we hypothesized that modifications to the large subunit of RNAP II occur after HSV-2 infection, as observed after HSV-1 infection (Chapter 3).

To determine whether HSV-2 induced modifications to RNAP II, I analyzed the large subunit of RNAP II by Western blotting. Vero cells were mock infected or infected with HSV-2 (strain G, American Type Culture Collection, Rockville, Maryland) or HSV-1 (KOS1.1). Cell extracts were harvested at various times, total proteins were separated on SDS-PAGE gels, and immunoblotting was performed with a mixture of antibodies that recognize the large subunit of RNAP II, 8WG16 and ARNA3 (Fig. 8.1). Mock infected cells contained both IIo and IIa (Fig. 8.1, lane 2). Infection with HSV-2 resulted in the loss of the IIo and IIa forms and the appearance of the intermediately migrating form, IIi, at 4 and 7 hr (Fig. 8.1, lane 4 and 7). This occurred with similar kinetics to that observed in KOS1.1 infected Vero cells (Fig. 8.1, lane 3 and 6). Infection with HSV-2 at 7 hr displayed an apparent loss of the RNAP II large subunit protein (Fig. 8.1, lane 7), which has also been observed occasionally at later times in KOS1.1 infection (Fig. 3.3A, lanes 7 and 8), although not observed in this particular experiment (Fig. 8.1, lane 6). We have not investigated the nature of this apparent depletion in RNAP II large subunit during late times of infection.

These data indicate that HSV-2 modifies RNAP II in a similar fashion to modifications induced by HSV-1. We suspect that the shift from IIo and IIa to IIi is due to phosphorylation and not from proteolysis of the IIo protein. However, calf alkaline intestinal phosphatase (CIAP) experiments as described in Chapter 3 have not been conducted on HSV-2 infected cell extracts. HSV-1 induction of IIi is dependent on ICP22 and UL13 gene products (Chapter 5). We do not know if HSV-2 ICP22 and HSV-2 UL13 are also required for IIi formation after HSV-2 infection. Interestingly, the level of conservation between HSV-1 and HSV-2 ICP22 protein sequences is over 50% near the N-terminus (Kilvert, 1995, Unpublished data; Whitton and Clements, 1984). The conservation between UL13 protein sequences in HSV-1 and HSV-2 has not been determined. The HSV-1 UL13 is similar to the HSV-2 UL13 in molecular mass, and antibodies raised against HSV-1 UL13 efficiently recognize HSV-2 UL13 (Overton et al., 1992). This suggests that some identity exists between HSV-1 and HSV-2's UL13 proteins, implying that a similar function may be found between HSV-1 and HSV-2 ICP22 and between HSV-1 and HSV-2 UL13. The induction of RNAP IIi during HSV-2 infection has not been further characterized.

The observation that HSV-2 infection results in the modification of RNAP II is not entirely surprising as HSV-1 and HSV-2 are very closely related. The viruses share a high degree of DNA and protein sequence identity and are similar in their regulation of gene transcription (Roizman and Sears, 1990). Although we have not examined transcription in HSV-2 infected cells, modifications to the large subunit of RNAP II may be necessary for efficient viral transcription in HSV-2.

C. Varicella-zoster virus (VZV) infection results in RNAP II modifications

VZV, an α-herpesvirus, shares some DNA sequence identity with HSV-1, especially in the unique short segments and adjacent repeats. Many VZV proteins display a high degree of amino acid sequence identity with their HSV homologues (Davison and McGeoch, 1986; Davison and Scott, 1986) and may possibly share protein function. Each VZV gene has an HSV-1 counterpart. VZV has the ability to complement HSV-1 temperature sensitive mutants. The VZV homologues of the HSV-1 IE genes ICP4 and ICP27 complement some HSV-1 temperature sensitive mutants and allow them to replicate efficiently at the nonpermissive temperature of 39°C (Felser et al., 1987).

VZV gene transcription is regulated in a similar manner to HSV-1. Transcription of VZV genes occurs in the nucleus and is dependent on the host's RNAP II transcription apparatus. Viral gene transcription occurs as a temporal cascade and is regulated predominantly at the transcriptional level (Gelb, 1990). Because VZV and HSV-1 display similarities in terms of DNA and protein sequence identity and gene regulation, it is possible that VZV modifies the large subunit of RNAP II, as observed after HSV-1 infection.

To test if VZV modified RNAP II, I examined the large subunit of RNAP II by Western blotting. Dr. Bill Ruyechan and Dr. David Stevenson, State University of New York at Buffalo, Buffalo, NY, prepared cell extracts that were analyzed and shown in Fig. 8.2. Human melanoma (MeWos) cells were mock infected or infected with VZV or HSV-1 for various times. Cell extracts were prepared and analyzed by SDS-PAGE and immunoblotting with a mixture of antibodies directed against the large subunit of RNAP II, 8WG16 and ARNA3 (Fig. 8.2).

VZV infected cells differed in the amount of cytopathic effect (cpe) from infection to infection. Cpe refers to changes in the cell structure after virus infection. These include cell rounding, detachment from the substrate, cell lysis, syncytium development, and inclusion body formation (Knipe, 1990). At 1 d and 4 d post-infection with VZV, two separate samples were collected. One sample displayed high cpe, and the second sample exhibited low levels of cpe. These samples were infected at high and low MOIs respectively.

Mock infected MeWos cells contained both IIa and IIo, although this sample predominantly contained IIa (Fig. 8.2, lane 2). The relative amounts of IIa to IIo were similar in several different cell extracts prepared from mock infected cells (data not shown). Infection of MeWos cells with HSV-1 resulted in the loss of IIo and the appearance of IIi, especially apparent at later times in infection (Fig. 8.2, lane 6). The induction of IIi after KOS1.1 infection in MeWos cells was generally similar to that observed after KOS1.1 infection in other cells, including Vero, HeLa S3 and HEL 299 cells (Chapter 3).

After infection with VZV, IIi was induced, although to a lesser amount (Fig. 8.2, lanes 3, 4, 7, and 8) than after an HSV-1 infection (Fig. 8.2, lane 6). At 1 and 4 d post-infection, the extent of RNAP II modification was variable. At 1 d post-infection, in samples where the cpe in cells was low, IIi was induced to some degree (Fig. 8.2, lane 7), while in samples where the cpe in cells was high, IIi was barely detectable (Fig. 8.2, lane 8). Conversely, at 4 d post-infection, where the cpe in cells was high, there was the loss of IIo and IIa and the induction of IIi (Fig. 8.2, lane 4). However, at 4 d post-infection, where the cpe in cells was low, there was very little RNAP II large subunit modification (Fig. 8.2, lane 3). The modifications to RNAP II large subunit were often difficult to distinguish (this experiment and data not shown). The extent of RNAP II modifications after VZV infection do not appear to depend on the cpe observed in cells.

What has been consistently observed in multiple VZV infected samples (this experiment and data not shown) is that slight modifications in RNAP II large subunit are observed after infection. We have not further investigated these modifications, although we suspect that the RNAP II large subunit is altered by phosphorylation after VZV infection, as it is after infection with other α-herpesviruses such as HSV-1 and HSV-2. Whether the induction of IIi is dependent on VZV's ICP22 homologue of HSV-1, open reading frame (ORF) 63 protein, or VZV's UL13 homologue of HSV-1, ORF 47 protein, is unknown.

Indirect immunofluorescence experiments performed by Dr. Bill Ruyechan and Dr. David Stevenson, State University of New York at Buffalo, Buffalo, NY, show that after VZV infection in MeWos cells, RNAP II is dispersed throughout the nucleus, with some preferential localization into distinct nuclear foci. These specific regions in the nucleus were found to be areas of VZV ORF 29 protein localization (Ruyechan and Stevenson, 1995, Unpublished data). VZV ORF 29 protein is the HSV-1 homologue of ICP8 and is the major DNA binding protein of VZV (Kinchington et al., 1988). VZV ORF 29 protein specifically localizes to the nucleus, often at discrete nuclear foci. Because of the difficulty in obtaining a synchronous infection with VZV, the time of distribution or stage in infection after which VZV ORF 29 localizes into nuclear foci has not been determined (Kinchington et al., 1988). The recruitment of RNAP II large subunit into these distinct nuclear foci after VZV infection is not as complete as during

HSV-1 infection (Chapter 3); some RNAP II molecules are still present outside these specific nuclear foci (Ruyechan, 1995, Unpublished data).

These experiments suggest that VZV modifies the large subunit of RNAP II. In addition, after VZV infection, there is some specific localization of RNAP II to distinct nuclear foci, sites which may be similar to DNA replication compartments in HSV-1 infected cells. Modifications to RNAP II after VZV infection are not unlikely since VZV is a member of the α-herpesvirus family and shares many commonalties with HSV-1 in terms of the regulation of gene transcription. Alterations in the RNAP II large subunit after VZV infection appear to be subtle in comparison with HSV-1 infected cells. This suggests that the requirement for RNAP II_I and its function in a VZV infection may be different than in an HSV-1 infection. At present, the importance of RNAP II_I in a VZV infection is unknown.

D. RNAP II modifications are absent after human cytomegalovirus (HCMV) infection

HCMV is a member of the β-herpesvirus family. The β-herpesviruses express their IE genes at a similar rate and time after infection as the α-herpesviruses, but β-herpesviruses initiate DNA replication at a much slower rate. The replication cycle requires days as opposed to hours for maximum production of infectious virus. HCMV's genome is the largest of all herpesviruses and is approximately 240 kb in size (Stinski, 1990).

There is some DNA and protein sequence identity between HCMV and other human herpesviruses, although this identity appears limited. Overall, the HCMV DNA polymerase is approximately 24% similar in protein sequence compared to DNA polymerases of HSV-1 and EBV. Higher degrees of sequence identity occur in specific regions of the protein (Kouzarides et al., 1987).

Like HSV-1 and HSV-2, HCMV is dependent on the RNAP II transcription apparatus. HCMV gene transcription occurs as a temporal cascade and is regulated primarily at the transcriptional level (Stinski, 1990).

To determine whether HCMV induced RNAP II modifications, I analyzed the large subunit of RNAP II by Western blotting. Dr. Alberto Severini, University of Alberta, Edmonton, Alberta, prepared cell extracts analyzed in Fig. 8.3. HEL 299 cells were mock infected or infected with HSV-1 (KOS1.1) or HCMV (strain AD169) for various times. Cells were harvested, total proteins were separated by SDS-PAGE, and immunoblotting was performed with antibodies directed against the RNAP II large subunit, ARNA3 (Fig. 8.3A) or 8WG16 (Fig. 8.3B).

Both IIa and IIo were present in mock infected cells, as detected by ARNA3 (Fig. 8.3A, lane 3), which recognizes all forms of the RNAP II large subunit. When a duplicate blot was probed with 8WG16, IIa was detected but not IIo (Fig. 8.3B, lane 3), because 8WG16 only recognizes incompletely phosphorylated forms of the RNAP II large subunit (Fig. 3.3, 3.4 and 3.5). In KOS1.1 infected cells, there was a loss of IIo and IIa and the appearance of IIi (Fig. 8.3A, lane 2), as detected with ARNA3. The induction of IIi was also observed when the KOS1.1 infected sample was probed with 8WG16 (Fig. 8.3B, lane 2). These results confirm what has been observed previously (Chapter 3).

HCMV infection resulted in no detectable changes to the mobility of the RNAP II large subunit on SDS-PAGE gels (Fig. 8.3A and 8.3B, lanes 4 to 9). Variable amounts of the RNAP II large subunit were observed in each lane, because the cell extracts were prepared at different concentrations (cell number/volume). Samples harvested at later post-infection times were more concentrated (Fig. 8.3A and 51B, lanes 7 to 9) than samples prepared at earlier post-infection times (Fig. 8.3A and 51B, lanes 4 to 6).

The samples were not tested to ensure that the cells were efficiently infected with HCMV. However, from the preliminary data presented, there are no detectable modifications in the large subunit of RNAP II after HCMV infection.

E. RNAP II modifications are absent after human herpes virus type 6 (HHV-6) infection

HHV-6 is another member of herpesvirus family, which shares most similarities with β-herpesviruses. The relatedness of HHV-6 to other herpesviruses has been examined by cross-hybridization studies and DNA sequencing experiments. HHV-6 shares DNA sequence identity in some regions of its genome with HCMV (Efstathious et al., 1988). Moreover, the predicted protein sequences from HHV-6 genes resemble those from HCMV genes (Lawrence et al., 1990).

HHV-6 transcription is thought to be similar to other herpesviruses in that the virus is dependent on the host RNAP II machinery for transcription of its genes. Furthermore, viral gene transcription is coordinated in a temporal cascade (Caserta and Hall, 1993). At present, limited information is available about how this cascade is regulated.

We did not expect to observe alterations in RNAP II after infection with HHV-6, since HCMV, which is the closest known relative of HHV-6, did not modify RNAP II. To test this prediction, I analyzed the large subunit of RNAP II in HHV-6 infected T lymphoblastoid (HSB-2) cells by Western blotting. Dr. Lung-Ji Chang, University of Alberta, Edmonton, Alberta, prepared cell extracts from HSB-2 cells that were uninfected or infected with HHV-6 for various times. Total proteins were separated and analyzed by SDS-PAGE and immunoblotting with antibodies recognizing the RNAP II large subunit, ARNA3 (Fig. 8.4A) or 8WG16 (Fig. 8.4B).

Uninfected HSB-2 cells contained IIa and IIo, as shown in the ARNA3 immunoblot (Fig. 8.4A, lane 4). When the same sample was probed with 8WG16, IIa and small amounts of incompletely phosphorylated RNAP II large subunit were observed (Fig. 8.4B, lane 4). The incompletely phosphorylated form migrated slightly slower than IIi produced in KOS1.1 infected Vero cells (Fig. 8.4B, lane 3), and may be the result of dephosphorylation of IIo during extract preparation. This is a possible explanation because cell extracts were prepared in the absence of phosphatase inhibitors. 8WG16 detects this incompletely phosphorylated form of RNAP II in uninfected HSB-2 cells, whereas ARNA3 does not.

After HHV-6 infection, there were no detectable changes in the phosphorylation of RNAP II large subunit, as observed in the ARNA3 immunoblot (Fig. 8.4A, lanes 5 to 7). IIa and IIo were retained as in uninfected cells (Fig. 8.4A, lane 4) and no IIi was induced. When the samples were probed with 8WG16, no changes in the phosphorylation state of the large subunit were observed (Fig. 8.4B, lanes 5 to 7), as compared to uninfected cells (Fig. 8.4B, lane 4). In the 8WG16 immunoblot, the same forms of RNAP II large subunit were present before and after HHV-6 infection (Fig. 8.4B, lanes 4 to 7). There were varied amounts of RNAP II observed at different times of infection because the extracts were prepared at different concentrations (cell number/volume). The sample prepared at 110 hr post-infection was less concentrated (Fig. 8.4A and 8.4B, lanes 7) than samples prepared at earlier times in infection (Fig. 8.4A and 8.4B, lanes 5 and 6).

Whether the HSB-2 cells were efficiently infected with HHV-6 was not determined. Western blotting experiments examining the production of the envelope protein gp82 and the early phosphoprotein p41 in the HHV-6 infected extracts were inconclusive (data not shown). The antibodies recognizing gp82 and p41 gave high background signals when used to probe HHV-6 infected cell extracts. From our preliminary data, there appears to be no detectable modifications in the large subunit of RNAP II after HHV-6 infection. HHV-6, like HCMV, may not require IIi for efficient viral transcription.

F. RNAP II modifications are absent after poxvirus infection

Poxviruses are large, brick-shaped viruses that are 300 to 450 nm by 170 to 260 nm in size. The viruses are enveloped and the genome consists of a double-stranded DNA molecule between 130 to 280 kb (Murphy and Kingsbury, 1990). Poxviruses are the only known family of DNA viruses that propagate entirely in the cytoplasm of eukaryotic cells and are largely self-sufficient. The virus encodes most, if not all, the enzymes and factors required for gene transcription and DNA replication. For example, poxviruses encode their own DNA polymerase and DNA-dependent RNAP (Moss et al., 1991). Poxvirus RNAP is a multisubunit enzyme of approximately 500 kDa and consists of two large and many smaller subunits, which resemble their eukaryotic counterparts (Baroudy and Moss, 1980). The largest subunit of the poxvirus RNAP shares some similarities with the large subunit of eukaryotic and prokaryotic RNAPs, but poxvirus RNAP lacks the CTD tail (Broyles and Moss, 1986).

Transcription of the poxvirus gene products are temporally regulated. There are at least three classes of genes, expressed in a cascade fashion. Early transcription begins immediately after virus entry into the cytoplasm. Remarkably, all the proteins necessary for transcription of the early class of genes are packaged in the virion particle. The subsequent synthesis of early viral proteins, one of them being DNA polymerase, results in DNA replication, after which intermediate and late gene expression ensues (reviewed in Moss, 1990; Moss et al., 1991). Cellular transcription and cellular translation are thought to be shut off after infection, although little is known about how this process occurs.

Vaccinia virus (VV) is a prototype member of the poxvirus family, and is part of the orthopoxvirus subfamily. We were interested in analyzing the large subunit of host RNAP II and determining whether there were changes to the abundance or phosphorylation of the protein after VV infection. Previous studies suggest that cellular RNAP II may be involved in the transcription of viral genes because it associates with virosomes, cytoplasmic regions that accumulate viral proteins, and is possibly packaged in virus particles (Morrison and Moyer, 1986). However, the role of host RNAP II in expression in poxvirus genes has not been clearly demonstrated.

To test whether VV infection resulted in modifications to host RNAP II, I examined the large subunit of RNAP II by Western blotting. HeLa S3 cells were mock infected, uninfected or infected with HSV-1 (KOS1.1) or VV (strain WR, Wyeth Reserve). Cells were harvested at various post-infection times and extracts were analyzed by SDS-PAGE and immunoblotting with antibodies directed against the large subunit of RNAP II, 8WG16 and ARNA3 (Fig. 8.5).

Mock infected and uninfected cells contained IIo and IIa (Fig. 8.5, lanes 2 and 4). KOS1.1 infection resulted in a loss of the IIa and IIo forms and an induction of the IIi form (Fig. 8.5, lane 3). After infection with VV, there were no detectable changes in the phosphorylation of RNAP II large subunit (Fig. 8.5, lanes 5 to 8): IIa and IIo were retained and IIi was not induced. At 16 hr post-infection, there was an apparent loss of the RNAP II large subunit (Fig. 8.5, lane 9). The overall depletion of the large subunit of RNAP II has been observed previously at late times after HSV-1 infections

(Fig. 3.3A, lanes 7 and 8). We have not further investigated the reasons for the loss of protein observed after infection. These data indicate that after VV infection, there are no detectable changes in the phosphorylation of the RNAP II large subunit.

The same VV stock used in our experiments was tested for its ability to infect HeLa S3 cells (Ellison, 1995, Unpublished data). HeLa S3 cells were infected with VV for various post-infection times. Cell extracts were prepared and the production of the viral uracil DNA glycosylase was examined by Western blotting. The presence of this protein demonstrated that the VV stocks were efficient in infecting HeLa S3 cells (Ellison, 1995, Unpublished data).

The localization of RNAP II after VV infection was examined (data not shown). Vero or baby green monkey kidney cells were mock infected or infected with VV for 5 hr. The cells were fixed and stained with the primary RNAP II large subunit antibody, 8WG16 or ARNA3, and the secondary GAMR antibody. In mock infected cells, RNAP II was dispersed throughout the nucleus, with exclusion from the nucleoli. After infection with VV, high degrees of cytopathic effects were observed. These include cell rounding and synctium formation. Staining of RNAP II large subunit was present throughout the cell in both the cytoplasm and nucleus. Our results support previous reports that the large subunit of RNAP II is translocated into the cytoplasm after rabbit poxvirus or VV infection (Morrison and Moyer, 1986; Wilton and Dales, 1986).

These data indicate that RNAP II large subunit may not be modified by phosphorylation after infection with VV, a member of the poxvirus family. This result is not unanticipated since poxviruses utilize their own RNAP to transcribe viral genes (Moss, 1990; Moss et al., 1991). Although the localization of RNAP II after poxvirus infection is altered (our experiments and Morrison and Moyer, 1986; Wilton and Dales, 1986), modifications to host RNAP II phosphorylation do not accompany these changes. Other studies suggest that host RNAP II plays a role in the transcription of viral genes because the protein is associated with virosomes and is packaged into the virus particle (Morrison and Moyer, 1986). Modifications to host RNAP II may not be required for RNAP II's association with virosomes and it subsequent packaging into the virus particle.

G. Summary

Modifications to the large subunit of RNAP II are observed following infection with all three of the human α-herpesviruses. HSV-2, like HSV-1, causes the depletion of IIa and IIo and the induction of IIi. VZV also modifies the large subunit of RNAP II although to a lesser extent. The extent of large subunit modifications correlate with the degree of genetic relatedness between the viruses.

Other herpesviruses such as HHV-6 and HCMV do not appear to aberrantly modify RNAP II by phosphorylation. The β-herpesviruses are similar to the α-herpesviruses in that both viruses are dependent on the host RNAP II transcription apparatus for transcription of viral genes and both viruses express their genes in a temporal fashion. However, modifications to the large subunit of RNAP II are not observed after HCMV or HHV-6 infection. Therefore, RNAP II modifications may not be required for the efficient transcription of β-herpesvirus genes.

VV infection does not appear to modify the large subunit of host RNAP II. Although poxviruses and α-herpesviruses share some similarities, in that both viruses contain DNA genomes and are enveloped, the viruses differ in the manner in which they transcribe their own genes. Although no direct evidence exists that host RNAP II transcribes poxvirus genes, some studies suggest that host RNAP II is important in poxvirus transcription (Silver et al., 1979; Morrison and Moyer, 1986; Wilton and Dales, 1986). Whatever role host RNAP II plays in VV transcription, modifications in the large subunit may not be of significance.

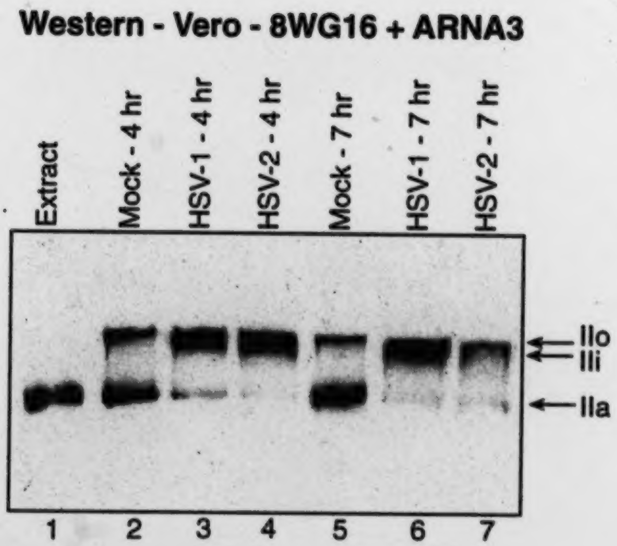

Western - Vero - 8WG16 + ARNA3
Extract
Mock - 4 hr
HSV-1 - 4 hr
HSV-2 - 4 hr
Mock - 7 hr
HSV-1 - 7 hr
HSV-2 - 7 hr
IIo
IIi
IIa
1
2
3
4
5
6
7

Figure 8.1. Western blot analysis of the RNAP II large subunit in HSV-2 infected Vero cells. Vero cells were mock infected (lanes 2 and 5) or infected with either HSV-1 (lanes 3 and 6) or HSV-2 (lanes 4 and 7) for the times indicated. Cells were harvested and lysed into Laemmli buffer. Whole cell extracts were separated on SDS-PAGE (6% acrylamide) gels and immunoblotted. The samples were probed with a mixture of both RNAP II large subunit antibodies, ARNA3 and 8WG16. Each lane contained extract from the same number of cells, except lane 1, which contained an aliquot of a nuclear *in vitro* transcription extract prepared from HeLa cells.

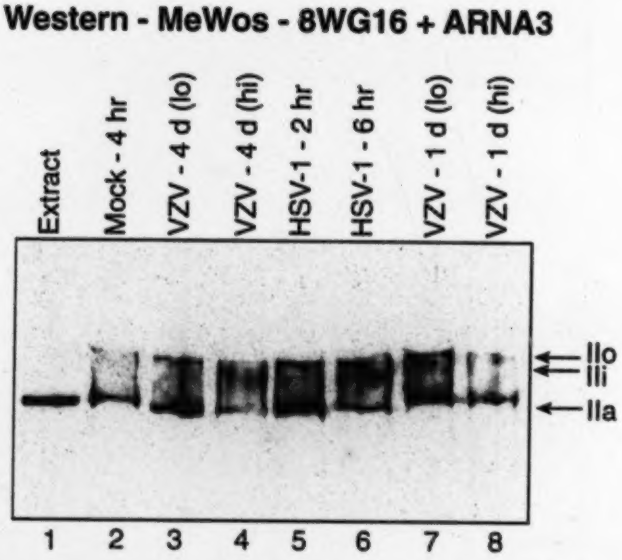
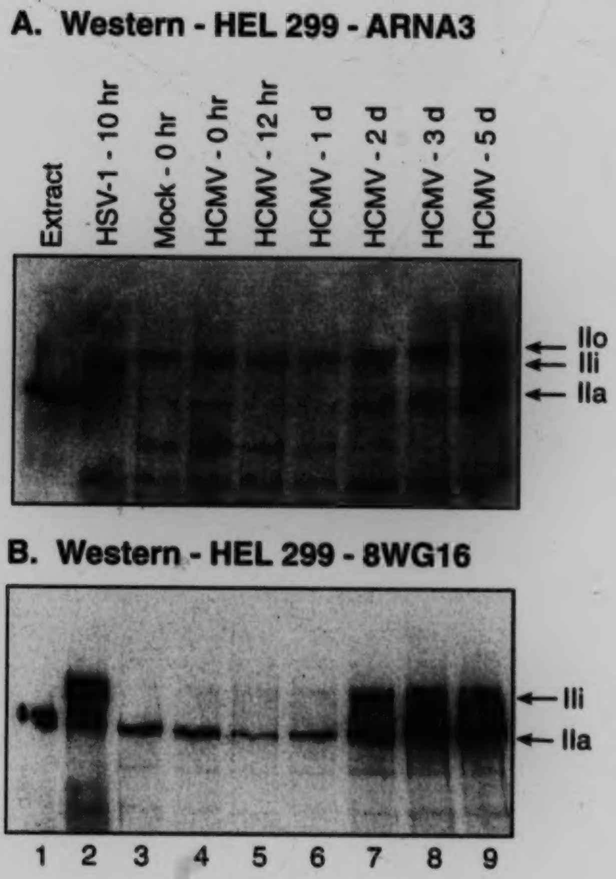

Western - MeWos - 8WG16 + ARNA3
Extract
Mock - 4 hr
VZV - 4 d (lo)
VZV - 4 d (hi)
HSV-1 - 2 hr
HSV-1 - 6 hr
VZV - 1 d (lo)
VZV - 1 d (hi)
IIo
IIi
IIa
1
2
3
4
5
6
7
8

Figure 8.2. Western blot analysis of the RNAP II large subunit in VZV infected MeWos cells. MeWos cells were mock infected (lane 2) or infected with either HSV-1 (lanes 5 and 6) or VZV (lanes 3, 4, 7 and 8) for the times indicated. VZV infected cells displayed high (lanes 4 and 8) or low (lanes 3 and 7) cytopathic effects. Whole cell extracts were provided by Dr. Bill Ruyechan and Dr. David Stevenson, University of Buffalo, Buffalo, NY. Western blotting was performed as described in the legend for Fig. 8.1. The samples were probed with a mixture of both RNAP II large subunit antibodies, ARNA3 and 8WG16. Lane 1 contained an aliquot of a nuclear *in vitro* transcription extract prepared from HeLa cells.

Figure 8.3. Western blot analysis of the RNAP II large subunit in HCMV infected HEL 299 cells. HEL 299 cells were mock infected (lanes 3) or infected with either HSV-1 (lanes 2) or HCMV (lanes 4 to 9) for the times indicated (0 hr to 5 d). Whole cell extracts were provided by Dr. Alberto Severini, University of Alberta, Edmonton, Alberta. Western blotting was performed as described in the legend for Fig. 8.1. Lanes 1 contained an aliquot of a nuclear *in vitro* transcription extract prepared from HeLa cells. The samples were probed with RNAP II large subunit antibodies ARNA3 (A) or 8WG16 (B). ARNA3 recognizes all forms of RNAP II large subunit, whereas 8WG16 recognizes the IIa and IIi subunits of RNAP II large subunit.



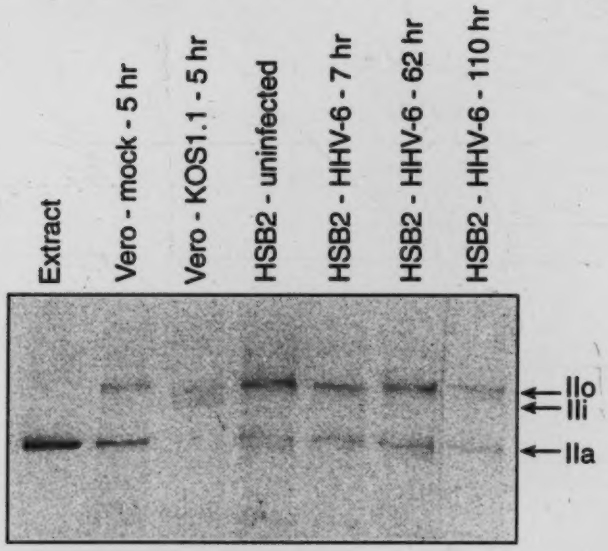
A. Western - HEL 299 - ARNA3

B. Western - HEL 299 - 8WG16

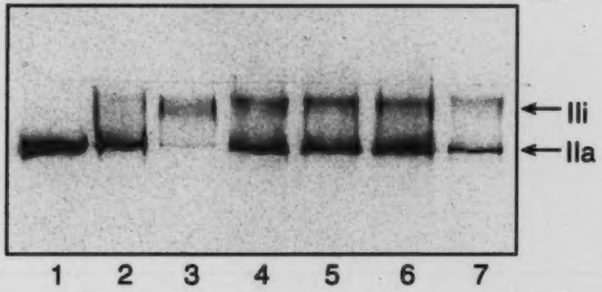
Extract
HSV-1 - 10 hr
Mock - 0 hr
HCMV - 0 hr
HCMV - 12 hr
HCMV - 1 d
HCMV - 2 d
HCMV - 3 d
HCMV - 5 d
IIo
IIi
IIa
IIi
IIa
1
2
3
4
5
6
7
8
9

Figure 8.4. Western blot analysis of the RNAP II large subunit in HHV-6 infected HSB-2 cells. Vero cells were mock infected (lanes 2) or HSV-1 (KOS1.1) infected (lanes 3), and T lymphoblastoid (HSB-2) cells were uninfected (lanes 4) or HHV-6 infected (lanes 5 to 7), for the times indicated. Whole cell extracts were provided by Dr. Lung-Ji Chang, University of Alberta, Edmonton, Alberta. Western blotting was performed as described in the legend for Fig. 8.1. Lanes 1 are of a nuclear *in vitro* transcription extract prepared from HeLa cells. The samples were probed with RNAP II large subunit antibodies ARNA3 (A) or 8WG16 (B). ARNA3 recognizes IIa, IIi, and IIo, whereas 8WG16 recognizes the IIa and IIi subunits of RNAP II large subunit.

A. Western - ARNA3


Extract
Vero - mock - 5 hr
Vero - KOS1.1 - 5 hr
HSB2 - uninfected
HSB2 - HHV-6 - 7 hr
HSB2 - HHV-6 - 62 hr
HSB2 - HHV-6 - 110 hr
IIo
IIi
IIa

B. Western - 8WG16


IIi
IIa
1
2
3
4
5
6
7

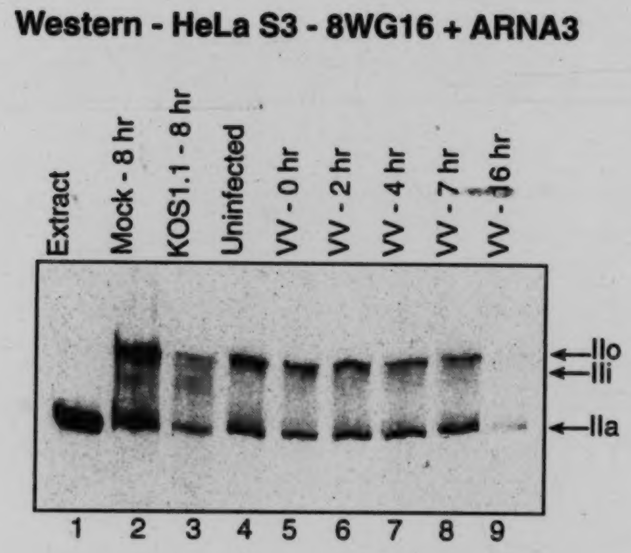

Western - HeLa S3 - 8WG16 + ARNA3
Extract
Mock - 8 hr
KOS1.1 - 8 hr
Uninfected
VV - 0 hr
VV - 2 hr
VV - 4 hr
VV - 7 hr
VV - 16 hr
IIo
IIi
IIa
1
2
3
4
5
6
7
8
9

Figure 8.5. Western blot analysis of the RNAP II large subunit in VV infected HeLa S3 cells. HeLa S3 cells were mock infected (lane 2), uninfected (lane 4), or infected with either HSV-1 (KOS1.1) (lane 3) or VV (strain WR) (lanes 5 to 9) for the times indicated. Western blotting was performed as described in the legend for Fig. 8.1. Each lane contained extract from the same number of cells, except lane 1, which contained an aliquot of a nuclear *in vitro* transcription extract prepared from HeLa cells. The samples were probed with a mixture of RNAP II large subunit antibodies ARNA3 and 8WG16.

CHAPTER NINE. CHARACTERIZATION OF HSV-1 FLAG ICP22 VIRUS AND STABLY TRANSFECTED HELA S3 FLAG ICP22 CELL LINES

A. Introduction

The repression of host transcription and the regulated activation of HSV-1 transcription may involve sequence independent mechanisms such as modifications to RNAP II transcription apparatus, alterations in DNA conformation, and localization of viral proteins into specialized nuclear compartments (Smiley et al., 1991). We have previously found that after HSV-1 infection, RNAP II is rapidly and aberrantly phosphorylated (Chapter 3). This modification is dependent on the presence of the IE protein ICP22 and the viral kinase UL13 (Chapters 4 and 5). The mechanism by which ICP22 and UL13 induce RNAP II modifications is unknown. There is evidence indicating that ICP22 and UL13 function in similar pathways. ICP22 and UL13 mutant viruses display similar cell type dependencies for growth (Purves et al., 1993). Both ICP22 and UL13 are required for efficient viral transcription in some cell lines (Chapter 5).

In order to delineate the biochemical pathway by which ICP22 functions and to characterize any interactions ICP22 may have with cellular or viral proteins, we required an ICP22 antibody. Although a polyclonal antibody to ICP22 (R77) (Ackermann et al., 1985) exists, we have found that it is largely nonspecific and cross-reacts with many cellular and viral proteins on Western blots and in immunofluorescence studies. Therefore, we tagged ICP22 with a FLAG epitope, which is recognized by the commercially available M2 antibody. An HSV-1 mutant virus containing FLAG ICP22 (F-ICP22), designated as F22, was constructed in order to analyze the ICP22 protein after infection. In addition, HeLa S3 cells were stably transfected with the F-ICP22 gene (HeLa S3 F-ICP22) in order to study RNAP II modifications in the presence of F-ICP22 alone.

B. Construction of the HSV-1 FLAG ICP22 virus F22

In order to analyze ICP22, an eight amino acid (DYKDDDDK) FLAG tag (Eastman Kodak Company) was inserted near the N-terminus of the ICP22 protein (Fig. 9.1). The FLAG ICP22 (F-ICP22) fusion was constructed by inserting a synthetic double-stranded oligonucleotide encoding the FLAG epitope in frame into the cloned HSV-1 ICP22 gene, 24 bp downstream of the ATG translation start site of ICP22. Cloning was performed by Alison Kilvert, University of Alberta, Edmonton, Alberta.

We have chosen to utilize the FLAG marker peptide in our experiments for a variety of reasons. Firstly, the M2 antibody, which specifically recognizes the FLAG epitope, appears to have little, if any, cross-reaction with mammalian or viral proteins (Eastman Kodak Company; data not shown). Secondly, the FLAG octapeptide is small and is likely to have minimal effects on ICP22 conformation or function. Thirdly, the FLAG amino acid sequence has high surface probability because of its hydrophilic nature. This enables the M2 antibody to easily recognize the FLAG epitope within the context of the heterologous protein. Fourthly, there are many well developed applications for FLAG fusion proteins. For instance, FLAG fusion proteins have been used to study protein localization, protein-protein interactions and protein DNA interactions (Eastman Kodak Company; Hopp et al., 1988; Chiang et al., 1993; Chiang and Roeder, 1995).

An HSV-1 recombinant virus containing the F-ICP22 gene was constructed by Stephen Rice and Vivian Leong (Lam), University of Alberta, Edmonton, Alberta. DNA containing F-ICP22 coding sequences was used for marker transfer with DNA from *d*22, an ICP22 null mutant. Vero cells were transfected with F-ICP22 and *d*22 DNA and recombinant viruses containing F-ICP22 were screened, plaque purified and characterized. We have designated the HSV-1 recombinant virus containing F-ICP22 as F22. The genome of F22 is similar to that of WT HSV-1 except that F22 contains the FLAG coding sequences near the N terminus of the ICP22 gene.

C. The large subunit of RNAP II is modified in F22 infected cells

It is important to determine that the FLAG epitope inserted near the N terminus of the ICP22 gene does not interfere with the growth of the virus or with ICP22 function. We characterized the F22 virus to ensure that F-ICP22 retained WT ICP22 functions.

The virus yields of F22 were compared to the virus yields of WT HSV-1 (KOS1.1) and ICP22 mutants, 22/*n*199 and *d*22 (Rice, 1996, Unpublished data). F22 and KOS1.1 grew to approximately 10^8 PFU/ml, whereas ICP22 mutants, 22/*n*199 and *d*22, grew to only 10^6 PFU/ml in HEL 299 cells. In addition, plaque morphology (size) of F22 and KOS1.1 was similar. These results indicate that F22 virus growth is as efficient as WT HSV-1 in HEL 299 cells, suggesting that F-ICP22 is similar to WT ICP22 in function.

As described in Chapters 3 and 4, RNAP II is aberrantly modified following WT HSV-1 infection, a phenomenon which is dependent on the IE protein ICP22. To ensure that the FLAG amino acid sequence had not disrupted the function of the ICP22 protein, we analyzed whether F22 was capable of inducing modifications in the large subunit of RNAP II. HeLa S3 cells were mock, KOS1.1, or F22 infected for various times and cell extracts were prepared. Total proteins were analyzed by SDS-PAGE and immunoblots were probed with RNAP II large subunit antibodies, 8WG16 and ARNA3 (Fig. 9.2).

IIa and IIo were present in mock infected cells (Fig. 9.2, lane 1). After KOS1.1 infection, IIi was induced at 4 , 6, and 8 hr post-infection (Fig. 9.2, lanes 3 to 5). Infection of cells with F22 from 4 to 8 hr post-infection also resulted in the induction of IIi (Fig. 9.2, lanes 7 to 9), similar to that observed in a KOS1.1 infection. There was a depletion of IIo and IIa after cells were infected with F22, especially evident at 6 and 8 hr post-infection (Fig. 9.2, lanes 8 and 9). In Fig. 9.2, lane 8 and 9, at later F22 infection times, there was the presence of immunoreactive material migrating slower than IIo. This material does not appear in all 6 and 8 hr F22 infected HeLa S3 cell extracts and may be specific to this particular Western blot (data not shown). We also found IIi induction and IIa and IIo depletion in F22 infected Vero and HEL 299 cells (data not shown). These data indicate that RNAP II modifications occur in F22 infected cells as efficiently as in KOS1.1 infected cells. Therefore, the presence of the FLAG epitope near the N terminus of ICP22 does not appear to disrupt ICP22 function with regards to either RNAP II modifications or virus growth efficiency.

D. Viral transcription in F22 infected cells

Previous experiments demonstrate that ICP22 is required for efficient viral transcription in HEL 299 cells but not in Vero cells (Chapter 5; Rice et al., 1995). To ensure that the FLAG amino acid sequence had not disrupted the viral transcription functions of the ICP22 protein, I performed nuclear run-on transcription assays. I compared viral transcription patterns in nuclei prepared from WT HSV-1 (KOS1.1), ICP22 mutant (22/*n*199), or F22 infected HEL 299 cells at 3, 6 and 9 hr post-infection. An equal number of nuclei per sample was analyzed. RNA transcripts initiated *in vivo* were elongated *in vitro* in the presence of ^{32}P-UTP, and nascent radiolabeled transcripts were hybridized to single-stranded DNA probes complementary to either specific sense (S) viral mRNAs or antisense (AS) RNAs from the same regions. The probes used detected transcription of two IE genes (ICP4 and ICP27), two DE genes (ICP8 and polymerase [pol]), and three L genes (VP16, gC, and UL36).

The WT KOS1.1 transcription pattern (Fig. 9.3A) was similar to that observed in previous nuclear run-on assays (Chapter 5; Godowski and Knipe, 1986; Weinheimer and McKnight, 1987; Rice et al., 1995). At 3 hr post-infection, specific sense transcription occurred and by 6 hr post-infection, high levels of IE, DE and L sense transcription were observed. At both 6 and 9 hr post-infection, there was antisense transcription in all regions of the viral genome, a phenomenon which appears to be contingent upon active viral DNA replication (Chapter 5; Godowski and Knipe, 1986; Weinheimer and McKnight, 1987; Rice et al., 1995).

The 22/*n*199 transcription pattern in HEL 299 cells (Fig. 9.3B) was similar to that observed in previous nuclear run-on assays (Chapter 5; Rice et al., 1995). Sense transcription in all three gene classes was reduced, especially at 6 and 9 hr post-infection. In addition, there was less antisense transcription at these time points.

The F22 transcription pattern (Fig. 9.3C) appeared different from the KOS1.1 (Fig. 9.3A) and 22/*n*199 (Fig. 9.3B) transcription patterns in HEL 299 cells. Specific sense transcription occurred in all regions of the genome at 3 hr and increased to high levels at 6 and 9 hr post-infection. There were also high levels of antisense transcription observed at 6 and 9 hr post-infection. Sense and antisense transcription of IE, DE and L genes appeared to be greater in

F22 infected cells, as compared to KOS1.1 and 22/*n*199 infected cells at 3, 6 or 9 hr post-infection.

To quantitate transcription in HEL 299 cells infected with KOS1.1, 22/*n*199, and F22, the signals from the nuclear run-on experiment shown in Fig. 9.3 were analyzed by PhosphorImager, focusing on the ICP27 (Fig. 9.4A and 9.5A), ICP8 (Fig. 9.4B and 9.5B), and gC (Fig. 9.4C and 9.5C) genes as representative members of the IE, DE, and L gene classes respectively. Interestingly, the level of ICP8 sense transcription at 3 hr post-infection in KOS1.1 infected HEL 299 cells was higher in this particular experiment (Fig. 9.4B) as compared to previous experiments (Fig. 5.8B). Sense transcription was reduced in all three genes at 6 and 9 hr post-infection in 22/*n*199 infected cells, compared with that of KOS1.1 or F22 infected cells (Fig. 9.4). Sense transcription was higher in all three genes at 3 and 6 hr post-infection in F22 infected cells, compared with that of KOS1.1 or 22/*n*199 infected cells (Fig. 9.4). The sense transcription of ICP27 and ICP8 at 9 hr post-infection was similar in both F22 and KOS1.1 infected cells (Fig. 9.4A and 9.4B). However, at 9 hr post-infection, the sense transcription of gC was higher in a F22 infection, as compared to a KOS1.1 or 22/*n*199 infection (Fig. 9.4C). Sense transcription of the gC gene at 9 hr in F22 infected cells was approximately one and a half-fold higher than in KOS1.1 infected cells.

There were also differences in the levels of antisense transcription in KOS1.1, 22/*n*199, and F22 infected cells. In all three genes, antisense transcription levels at 9 hr were lower in 22/*n*199 infected cells compared to KOS1.1 or F22 infected cells (Fig. 9.5). At 9 hr post-infection, antisense transcription in all three genes was higher in F22 infected cells, as compared to KOS1.1 or 22/*n*199 infected cells (Fig. 9.5). The levels of antisense transcription in F22 infected cells at 9 hr in ICP27 (Fig. 9.5A), ICP8 (Fig. 9.5B) and gC (Fig. 9.5C) were approximately one and a half-fold, one and a half-fold, and twofold higher than in KOS1.1 infected cells respectively.

These results indicate that viral transcription in a F22 infection was generally more efficient than in a KOS1.1 or a 22/*n*199 infection. Because viral transcription levels are higher in F22 infected cells as compared to KOS1.1 infected cells, this suggests that the FLAG epitope fused to F-ICP22 may be improving the transcriptional activity of the virus. However, these results show that the FLAG epitope does not disrupt or mutate the F-ICP22 protein in such a way that the transcriptional activities of F-ICP22 are impaired.

The nuclear run-on assay comparing viral transcription in F22, KOS1.1, and 22/*n*199 infected HEL 299 cells was performed only once. This experiment needs to be repeated in order to verify our results. In addition, viral transcription in F22 infected Vero or HeLa S3 cells has not been examined.

E. FLAG ICP22 (F-ICP22) is a phosphorylated protein

To determine whether F22 produced full-length F-ICP22 protein and to examine the F-ICP22 protein expression profile in infected cells, I analyzed F-ICP22 by Western blotting. HeLa S3 cells were mock, WT HSV-1 (KOS1.1), or F22 infected and cell lysates were prepared at various post-infection times. Total proteins were separated on SDS-PAGE gels and immunoblotting was performed with the M2 antibody (Fig. 9.6). F-ICP22 protein was detected in F22 infected cells as early as 4 hr post-infection (Fig. 9.6, lanes 7 to 9). In contrast, mock and KOS1.1 infected cells contained no detectable F-ICP22 protein (Fig. 9.6, lanes 1 to 5). The F-ICP22 protein migrates similarly to WT ICP22, between 70 and 80 kDa (Purves and Roizman, 1992).

F-ICP22 was also observed in cell extracts prepared from Vero and HEL 299 cells infected with F22 (data not shown). The kinetics of F-ICP22 protein expression in Vero and HEL 299 cells was similar to that observed in HeLa S3 cells. Furthermore, similar protein expression of WT ICP22 has been observed in baby hamster kidney (BHK) cells (Purves et. al, 1993). No F-ICP22 was detected in Vero or HEL 299 cells that were mock or KOS1.1 infected.

Infection of HeLa S3 cells with F22 produced multiple forms of F-ICP22 (Fig. 9.6, lanes 7 to 9). At 4 hr post-infection, two forms of F-ICP22 were detected, whereas at 6 and 8 hr post-infection, three forms of F-ICP22 were observed. Multiple forms of F-ICP22 were also found in cell extracts prepared from F22 infected Vero and HEL 299 cells (data not shown). We suspect that these multiple bands detected by Western blotting with the M2 antibody represent phosphorylation variants of F-ICP22.

To confirm that multiple forms of F-ICP22 observed by Western blotting are phosphorylation variants of the same protein, I treated F22 infected cell extracts with calf intestinal alkaline phosphatase (CIAP). CIAP treatment of all forms of F-ICP22 at 4, 6, and 8 hr post-infection converted them to one nonphosphorylated form (Fig. 9.7, lanes 2, 4, and 6). These results indicate that the multiple forms of F-ICP22 observed in Western blotting are due to phosphorylation differences.

Five differently phosphorylated ICP22 species have been detected in WT HSV-1 infected human epidermoid carcinoma (HEp-1) and BHK cells (Purves and Roizman, 1992). In our experiments, we detect up to 3 distinct F-ICP22 phosphorylation variants in F22 infected HeLa S3 cells (Fig. 9.7, lanes 3 and 5). Differences in the number of F-ICP22 forms observed may be due to cell type specificities (Purves and Roizman, 1992). Alternatively, it is possible that only 3 forms of F-ICP22 are observed in F22 infected HeLa S3 cells because of the presence of the FLAG epitope fused to the ICP22 protein. The FLAG tag may interfere with the phosphorylation of the ICP22 protein even though the FLAG epitope is inserted at the amino acid 8 position of ICP22 which is distant from the predicted ICP22 serine and threonine phosphorylation sites between amino acids 38 to 118 and 296 to 385 (Purves and Roizman, 1992). Also, the resolution on our SDS-PAGE gels may not be sufficient to visualize all forms of F-ICP22.

In HeLa S3 cells, RNAP II modifications occur approximately 4 to 5 hr post-infection (Chapter 3), coincident with the increase in F-ICP22 phosphorylation observed between 4 and 6 hr post-infection. These two events may or may not be related. It is unknown whether efficient ICP22 phosphorylation is required for RNAP II modifications. ICP22 modifications may be required for the induction of IIi in infected cells as UL13 is necessary for efficient ICP22 phosphorylation and for RNAP II modifications (Chapter 5).

F. F-ICP22 localization in F22 infected HeLa S3 cells

To test whether F-ICP22 is specifically recruited into viral DNA replication compartments, I performed double immunofluorescence experiments. Vero cells were infected with F22 for 6 hr after which the cells were fixed and stained with the M2 antibody, which specifically recognizes the FLAG epitope, and 3-83, a polyclonal antibody recognizing ICP8 (Knipe et al., 1987) (Fig. 9.8). F-ICP22 showed nuclear staining with some bright speckles present throughout the nucleus (Fig. 9.8B). These speckles may represent specific aggregations of the F-ICP22 protein. Interestingly, some of these speckles appeared to be present in DNA replication compartments, as detected by ICP8 colocalization (Fig. 9.8C). There may also be some preferential F-ICP22 staining in DNA replication compartments since F-ICP22 staining is slightly stronger in these compartments.

Control experiments showed no cross-reaction of the M2 and 3-83 antibodies or of the two secondary antibodies, GAMR and DARF (data not shown). The staining patterns of 3-83 (Fig. 9.8C) showed some areas of staining which did not colocalize with F-ICP22 staining (Fig. 9.8B), as observed previously in Fig. 3.2.

These data indicate that F-ICP22 localizes to the nucleus during F22 infection. There may also be some preferential localization of F-ICP22 into viral DNA replication compartments. These observations are in agreement with previous results of Leopardi et al. (1997) showing ICP22 colocalization with ICP4 and RNAP II, proteins which are recruited into DNA replication compartments in WT HSV-1 infected cells. Additional ICP22 staining is observed in the nucleus but outside of the DNA replication compartments (Leopardi et al., 1997). The localization of ICP22 into DNA replication compartments appears to be dependent on UL13 since ICP22 is not efficiently localized into these compartments in UL13 mutant infected cells (Leopardi et al., 1997). Since ICP22 phosphorylation is dependent on UL13, these results suggest that proper ICP22 modifications may be required for its recruitment into viral nuclear compartments.

G. Stably transfected HeLa S3 F-ICP22 cell lines

To test whether RNAP II modifications occurred efficiently in the sole presence of F-ICP22, we expressed F-ICP22 in HeLa S3 cells using the tetracycline (tet)-VP16 inducible system. The F-ICP22 gene was first cloned into the pUHD 10-3 plasmid (Gossen and Bujard, 1992), which placed F-ICP22 under the control of the human cytomegalovirus (HCMV) minimal promoter and 7 copies of the tet-operator sequence (Gossen and Bujard, 1992). In *E. Coli*, the tet repressor protein binds to the tet operator sequence and represses transcription of the tet resistance operon. In the presence of tetracycline, tetracycline binds to the tet repressor and prevents the repressor from binding to the tet operator. As a result, the tet resistance operon is transcriptionally induced (Hillen and Wissmann, 1989). In the tet-VP16 inducible system, the tet repressor is fused to the activation domain of VP16, creating a tetracycline controlled activator that is capable of stimulating the tet resistance operon. In the presence of tetracycline, tetracycline binds to the tet repressor VP16 fusion protein, releasing it from operator binding. In the absence of tet-VP16 activator binding, the HCMV minimal promoter is inactive. In the absence of tetracycline, the tet repressor VP16 fusion protein is capable of binding to operator sequences and activating transcription from genes containing tet operators (Gossen and Bujard, 1992).

HeLa S3 cells lines were stably transfected with DNA encoding the tet-VP16 expression plasmid, creating cell line 33. Cell line 33 was tested for tet-VP16 expression by transient transfections with a plasmid containing ICP22 under the control of the HCMV minimal promoter and 7 copies of the tet-operator sequence (Kilvert, 1995, Unpublished data). Cell line 33 was then stably transfected with the F-ICP22 gene under the control of the HCMV minimal promoter and 7 copies of the tet operator. In the presence of tetracycline, tetracycline binds to the tet repressor VP16 fusion protein and F-ICP22 transcription should not occur. In the absence of tetracycline, the tet repressor VP16 fusion protein binds to the operator sequences and should stimulate transcription of the F-ICP22 gene.

To ensure that F-ICP22 DNA had been stably incorporated into F-ICP22 cell lines, I performed a Southern blot. DNA from each of the F-ICP22 cell lines was isolated, digested with *Hinc* II, and separated on an agarose gel. A diagram of the F- ICP22 insertion present in HeLa S3 genomic DNA is shown with labeled restriction enzyme sites in Fig. 9.9. The samples were subjected to Southern blotting using the radiolabeled ICP22 DNA probe (Fig. 9.9) and then visualized by autoradiography (Fig. 9.10).

Hybridization to the positive control, consisting of a 7.6 kb linearized DNA fragment containing F-ICP22, indicated that the ICP22 DNA probe used in the Southern blot was specifically recognizing ICP22 (Fig. 9.10, lane 1). A negative control contained DNA from cell line 33, the tet-VP16 expressing cell line, was also analyzed (data not shown). No bands were observed in the

Southern blot, indicating that the ICP22 DNA probe was not detecting a nonspecific DNA fragment present in cell line 33. No ICP22 positive bands were detected in cell lines 2, 5, 11, 12, 16, and 24 (Fig. 9.10, lanes 2, 5, 9, 10, 13, and 14). Cell lines 3, 9, 10, and 13 contained multiple copies of the F-ICP22 gene incorporated into the cellular genome, as indicated by the multiple DNA fragments recognized by the ICP22 DNA probe (Fig. 9.10, lanes 3, 7, 8, and 11). Cell lines 4, 8 and 30 may contain only one copy of the F-ICP22 gene incorporated into the cellular genome, as detected by the single DNA fragment recognized by the ICP22 DNA probe (Fig. 9.10, lanes 4, 6, and 15). It is possible that there was more than one site of incorporation of the F-ICP22 gene in these particular cell lines but only one band was observed in Southern blotting. Cell line 14 likely contained a deletion in the F-ICP22 gene because the DNA fragment detected in Southern blotting was smaller than the minimum 3.4 kb ICP22-*Hinc* II fragment detected by the ICP22 DNA probe (Fig. 9.10, lane 12). Cell lines 3, 9, and 13 also contained F-ICP22 inserts smaller in size than the intact ICP22 gene (Fig. 9.10, lanes 2, 7, 11, and 12).

To ensure that F-ICP22 mRNA was synthesized from the stably transfected F-ICP22 cells, I performed a Northern blot. Cell lines 3, 4, 8, 10, 13, and 30 which have at least one F-ICP22 gene incorporated into the HeLa S3 genome were induced for F-ICP22 expression in the absence of tetracycline for 24 hr. Total RNA was harvested from uninduced and induced cell lines and separated on an agarose formaldehyde gel. Northern blotting (Fig. 9.11) was performed and the blot was probed with the radiolabeled ICP22 DNA probe (Fig. 9.9).

A linearized 7.6 kb DNA fragment from a plasmid containing the F-ICP22 gene was used as a positive control and was recognized by the ICP22 DNA probe efficiently (Fig. 9.11, lane 1). Negative controls contained total RNA harvested from nontransfected HeLa S3 cells and cell line 33, the tet-VP16 expressing line (Fig. 9.11, lanes 2 to 4). No RNA from nontransfected HeLa S3 cells and cell line 33 cross-reacted with the ICP22 DNA probe. After induction, F-ICP22 mRNA was not detected in cell lines 8 and 13 (Fig. 9.11, lanes 10 and 14), despite the presence of F-ICP22 DNA in their genomes (Fig. 9.10, lanes 6 and 11). By visual inspection, cell lines 3, 4, and 10 showed a two- to threefold increase in F-ICP22 mRNA in induced cells (Fig. 9.10, lanes 6, 8 and 12). Cell line 30 showed no increase in F-ICP22 mRNA in induced cells

(Fig. 9.10, lanes 16). The ICP22 mRNA synthesized in uninduced cell lines may represent a certain amount of "leaky" gene expression. The induction F-ICP22 mRNA was not as significant as that reported previously for the tet-VP16 inducible system (Crameri and Stemmer, 1995). We have not investigated why certain cell lines did not express F-ICP22 mRNA even though they contained copies of the F-ICP22 gene incorporated into their genomes nor have we explored the reasons why induction did not increase the expression of ICP22 mRNA.

To test whether the stably transfected F-ICP22 cell lines expressed the F-ICP22 protein, I performed a Western blot. Cell lines 3, 4, 8, 9, 10, 13, and 30 which have the F-ICP22 gene incorporated into the HeLa S3 genome were induced for F-ICP22 expression in the absence of tetracycline for 24 hr. Cell lysates were prepared and proteins were separated on SDS-PAGE gels. Immunoblotting was conducted with the M2 antibody, which recognizes the FLAG epitope of F-ICP22 (Fig. 86A and 86B).

A nuclear extract of F22 infected HeLa S3 cells was used as a positive control to ensure that the M2 antibody specifically recognized the F-ICP22 protein (Fig. 9.12A and 9.12B, lane 1). No F-ICP22 protein was detected in uninduced and induced cell line 33, the tet-VP16 expressing cell line (Fig. 9.12A and 9.12B, lanes 2 and 3). No F-ICP22 protein was detected in cell lines 3, 4, 8, 9, 10, 13, and 30 either before or after induction (Fig. 9.12A, lanes 5, 7, and 9 and Fig. 9.12B, lanes 3, 5, 7 and 9), despite the presence of single or multiple copies of the F-ICP22 gene in their genomes (Fig. 9.10, lanes 3, 4, 6, 7, 8, 11, and 15). In cell lines 8 and 13, we did not expect F-ICP22 protein to be expressed after induction in the absence of tetracycline (Fig. 9.12A, lane 7 and Fig. 86B, lane 9) since F-ICP22 mRNA was not detected (Fig. 9.11, lanes 10 and 14). Surprisingly, F-ICP22 protein was not detected in cell lines 3, 4, 10, and 30 after induction (Fig. 9.12A, lanes 5 and 9 and Fig. 9.12B, lanes 3 and 7) even though F-ICP22 mRNA was synthesized (Fig. 9.11, lanes 6, 8, 12, and 16).

The lack of F-ICP22 protein observed after induction of cell lines 3, 4, 9, 10, and 30 may be due to a variety of reasons. It is possible that the induction of F-ICP22 in these cell lines may be inefficient and the levels of ICP22 mRNA synthesized are not sufficient for abundant amounts of protein to be produced. It is also possible that the ICP22 protein is toxic to cells and stably transfected HeLa S3 F-ICP22 cell lines that express F-ICP22 protein are not viable. Hence, only F-ICP22 non-expressing cells would be selected and isolated. It has been previously observed that mutant viruses, simultaneously defective in ICP4, ICP27 and ICP22 are less toxic to Vero and HEL cells (Wu et al., 1996). Furthermore, it is thought that ICP0, ICP27, and ICP22 encode cytopathogenic gene products, because transfection of each these genes into BHK and African green monkey kidney (CV-1) cells results in an inhibition of colony formation (Johnson et al., 1994). Because F-ICP22 protein is not expressed in these stably transfected cell lines, we are unable to determine whether RNAP II modifications occur in the sole presence of F-ICP22 protein.

H. Summary

I attempted to characterize the F22 virus and to ensure that F-ICP22 retained WT ICP22 functions in terms of RNAP II modifications and viral transcription. The incorporation of the FLAG epitope near the N terminus of ICP22 does not appear to compromise virus growth kinetics or destroy ICP22 function. F-ICP22 is capable of inducing RNAP II modifications and performing its viral transcription functions. The F22 virus may exhibit higher viral transcription rates than WT HSV-1.

By using F22, we have been able to examine the protein expression profile of F-ICP22. F-ICP22 is expressed as early as 4 hr post-infection and is modified extensively by phosphorylation. Increased phosphorylation of F-ICP22 especially occurs after 6 hr post-infection. Furthermore, the localization of F-ICP22 is nuclear with some preferential recruitment into DNA replication compartments. In Chapter 12, I will describe the use of the F22 virus to study ICP22 protein-protein interactions.

Stably transfected F-ICP22 cell lines which have single or multiple copies of the F-ICP22 gene incorporated in their genomes contain limited F-ICP22 mRNA expression before and after induction. Furthermore, these cell lines do not express any detectable F-ICP22 protein. It is possible that F-ICP22 is toxic to HeLa S3 cells and therefore F-ICP22 expressing cells have not been isolated. We are thus unable to determine if RNAP II modifications occur in the sole presence of F-ICP22 protein.

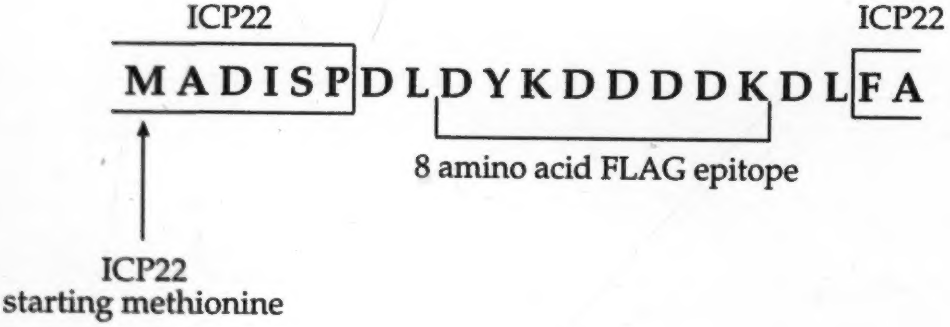

ICP22
ICP22
MADISP D L DYKDDDDK D L FA
8 amino acid FLAG epitope
ICP22
starting methionine

Figure 9.1. FLAG epitope inserted near the N terminus of the ICP22 protein. The FLAG epitope (DYKDDDDK) recognized by the M2 antibody is inserted near the N terminus, 8 amino acids downstream of the starting methionine, of the ICP22 protein. Cloning was performed by Alison Kilvert, University of Alberta, Edmonton, Alberta.

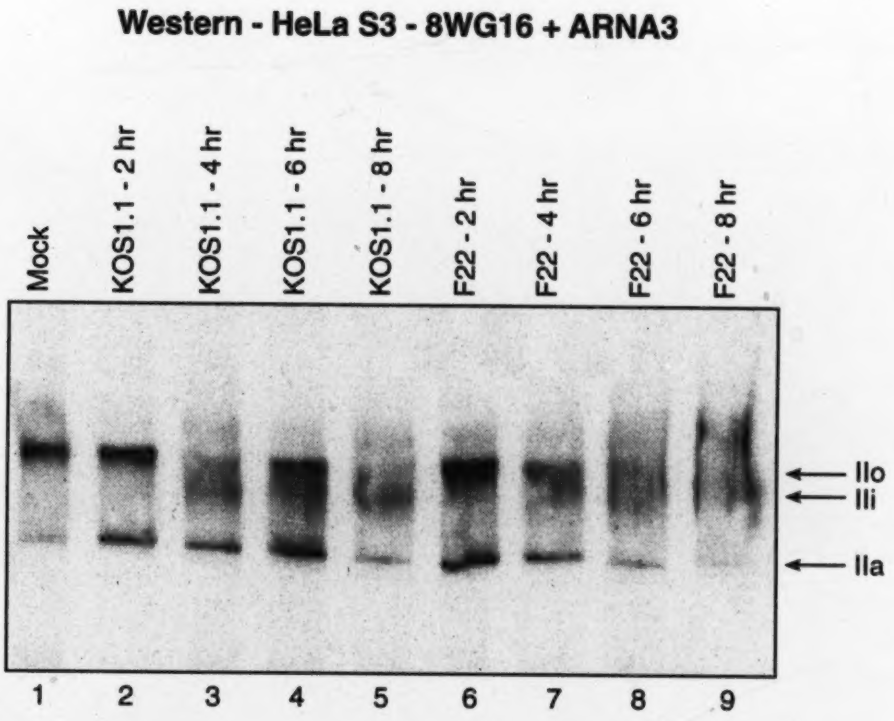

Western - HeLa S3 - 8WG16 + ARNA3
Mock
KOS1.1 - 2 hr
KOS1.1 - 4 hr
KOS1.1 - 6 hr
KOS1.1 - 8 hr
F22 - 2 hr
F22 - 4 hr
F22 - 6 hr
F22 - 8 hr
IIo
IIi
IIa
1
2
3
4
5
6
7
8
9

Figure 9.2. Western blot analysis of the RNAP II large subunit in F22 infected HeLa S3 cells. HeLa S3 cells were mock infected (lane 1) or infected with WT HSV-1 (KOS1.1) (lanes 2 to 5) or with the HSV-1 recombinant FLAG ICP22 virus (F22) (lanes 6 to 9) for the times indicated. Cells were harvested and lysed into Laemmli buffer. The whole cell extract was run on SDS-PAGE (6% acrylamide) gels, immunoblotted and probed with a mixture of RNAP II large subunit antibodies ARNA3 and 8WG16.

Figure 9.3. Nuclear run-on transcription analysis of viral gene transcription in infected HEL 299 cells. Vero cells were infected with HSV-1 (KOS1.1) (A), the HSV-1 ICP22 mutant 22/*n*199 (B), or the HSV-1 recombinant FLAG ICP22 virus (F22) (C) for the times indicated. Nuclei were isolated and transcription was allowed to proceed in the presence of ^{32}P-UTP as described in Chapter 2. RNA products from equal numbers of nuclei per sample were hybridized to immobilized single-stranded DNA probes that detect sense (S) or antisense (AS) transcripts arising from the IE genes ICP4 and ICP27, the DE genes ICP8 and DNA polymerase, and the L genes VP16, gC, and UL36. Single-stranded DNA of M13mp19 was included as a background hybridization control. Nuclear run-on transcription assays of mock infected cells yielded no hybridization to these probes (data not shown).

Nuclear Run-on - HEL 299 - Viral Transcription

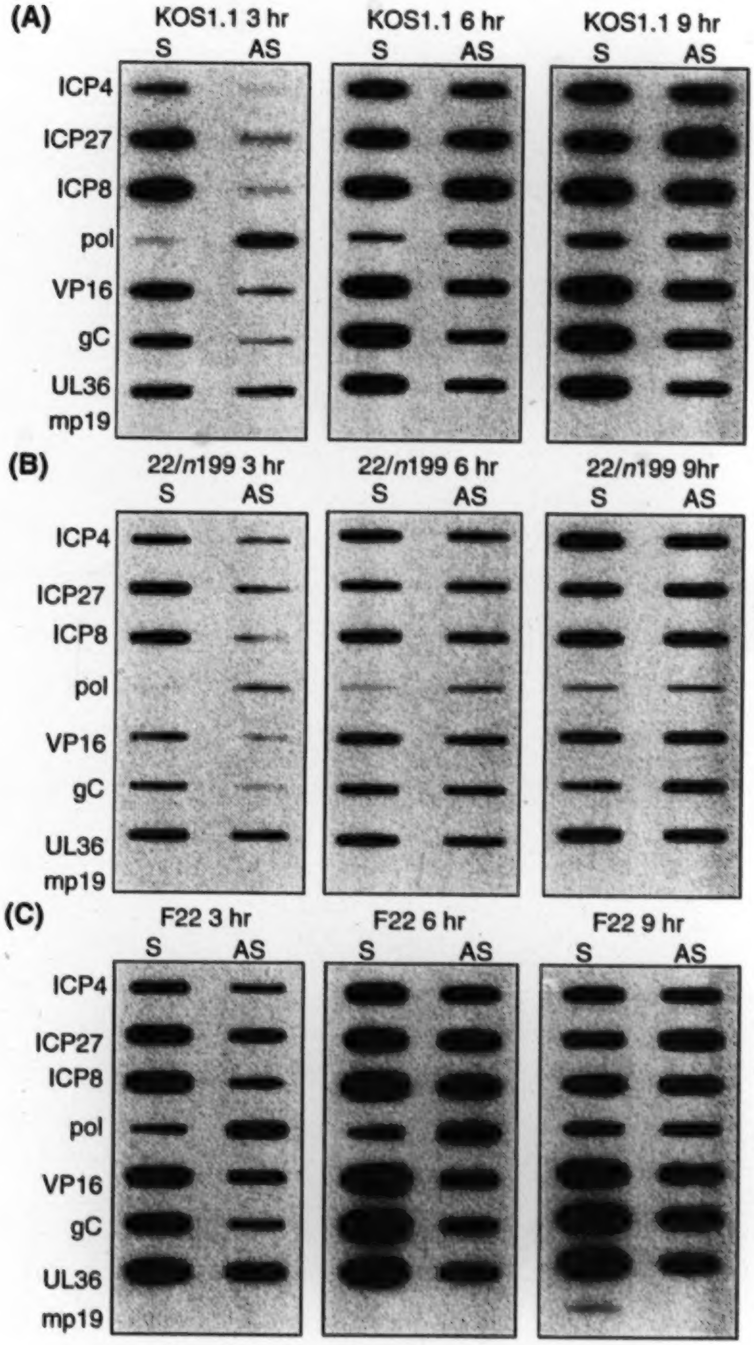
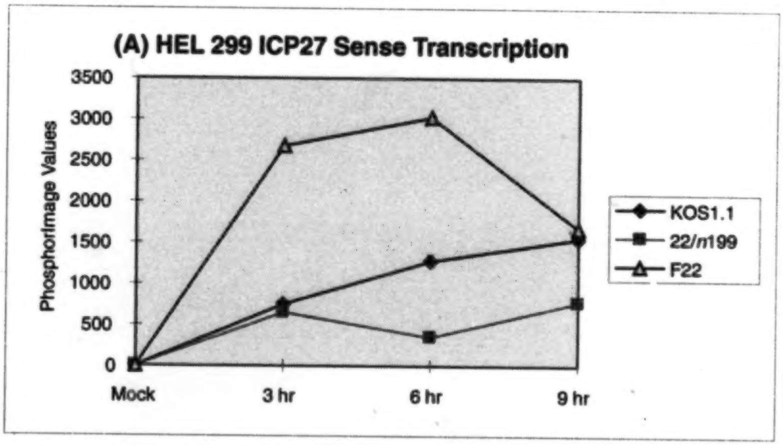
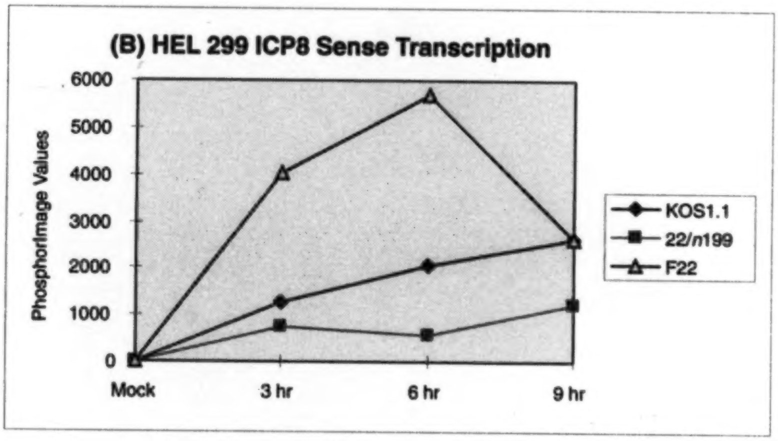

(A)
KOS1.1 3 hr
KOS1.1 6 hr
KOS1.1 9 hr
S
AS
ICP4
ICP27
ICP8
pol
VP16
gC
UL36
mp19
(B)
22/n199 3 hr
22/n199 6 hr
22/n199 9hr
S
AS
ICP4
ICP27
ICP8
pol
VP16
gC
UL36
mp19
(C)
F22 3 hr
F22 6 hr
F22 9 hr
S
AS
ICP4
ICP27
ICP8
pol
VP16
gC
UL36
mp19

Figure 9.4. Quantitation of sense (S) viral gene transcription in infected HEL 299 cells. The relative ^{32}P-hybridization signal on specific sense (S) gene probes obtained from nuclear run-on analysis of viral transcription in HEL 299 cells was quantitated by PhosphorImager analysis: top, ICP27; middle, ICP8; bottom, gC. The M13mp19 background control was subtracted from each of the values.


(A) HEL 299 ICP27 Sense Transcription


(B) HEL 299 ICP8 Sense Transcription

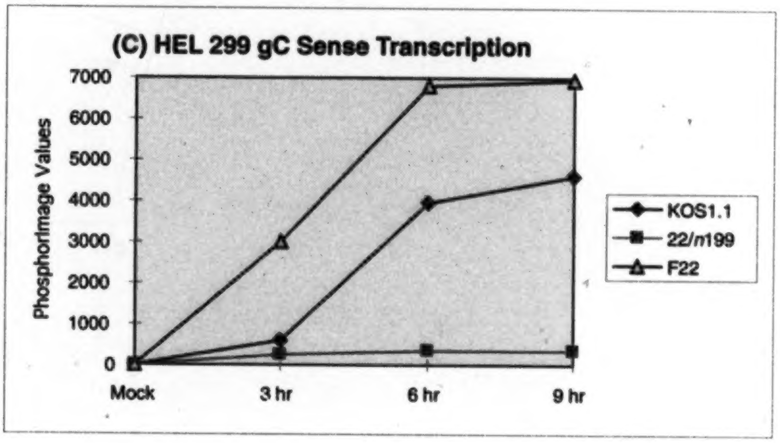
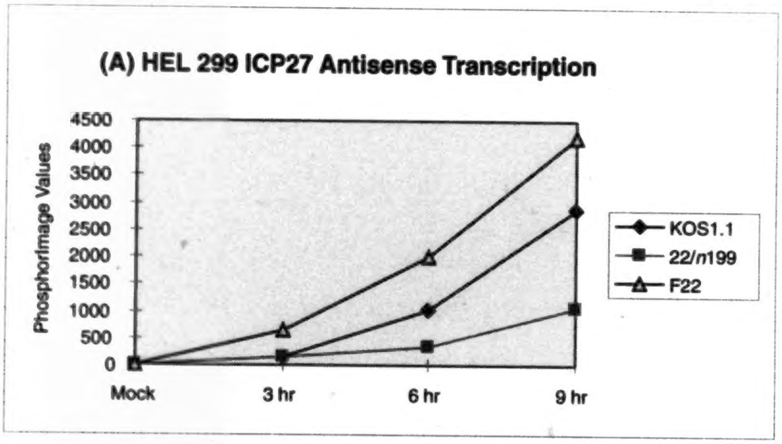
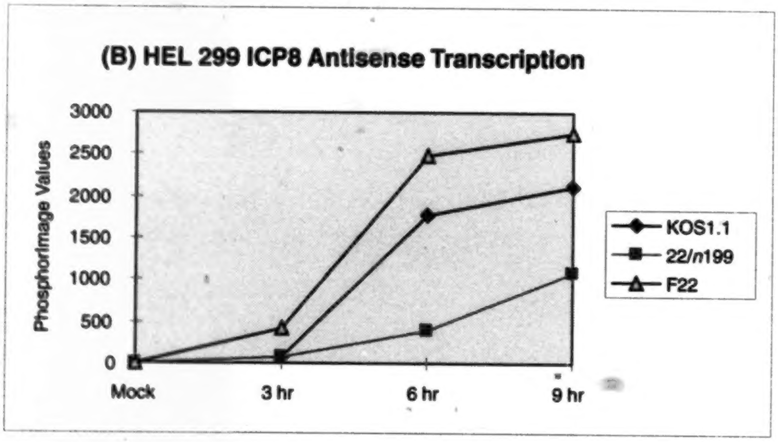
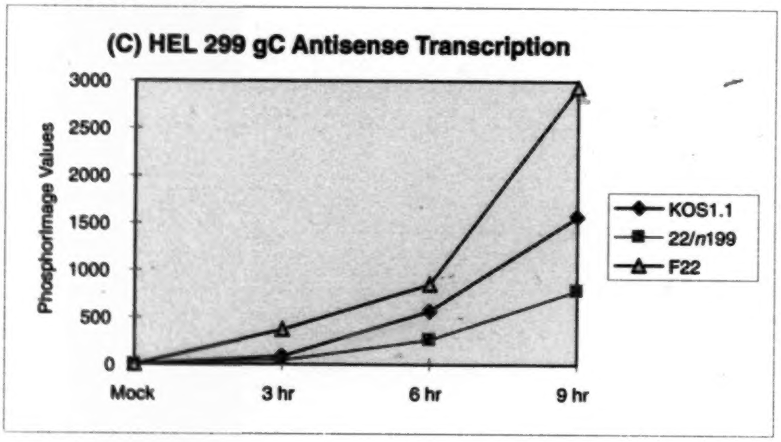

(C) HEL 299 gC Sense Transcription

Figure 9.5. Quantitation of antisense (AS) viral transcription in infected HEL 299 cells. The relative ^{32}P-hybridization signal on specific antisense (AS) probes obtained from nuclear run-on analysis of viral transcription in HEL 299 cells was quantitated by PhosphorImager analysis: top, ICP27; middle, ICP8; bottom, gC. The M13mp19 background control was subtracted from each of the values.


(A) HEL 299 ICP27 Antisense Transcription
Phosphorimage Values
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Mock
3 hr
6 hr
9 hr
KOS1.1
22/n199
F22


(B) HEL 299 ICP8 Antisense Transcription
Phosphorimage Values
3000
2500
2000
1500
1000
500
0
Mock
3 hr
6 hr
9 hr
KOS1.1
22/n199
F22


(C) HEL 299 gC Antisense Transcription
Phosphorimage Values
3000
2500
2000
1500
1000
500
0
Mock
3 hr
6 hr
9 hr
KOS1.1
22/n199
F22

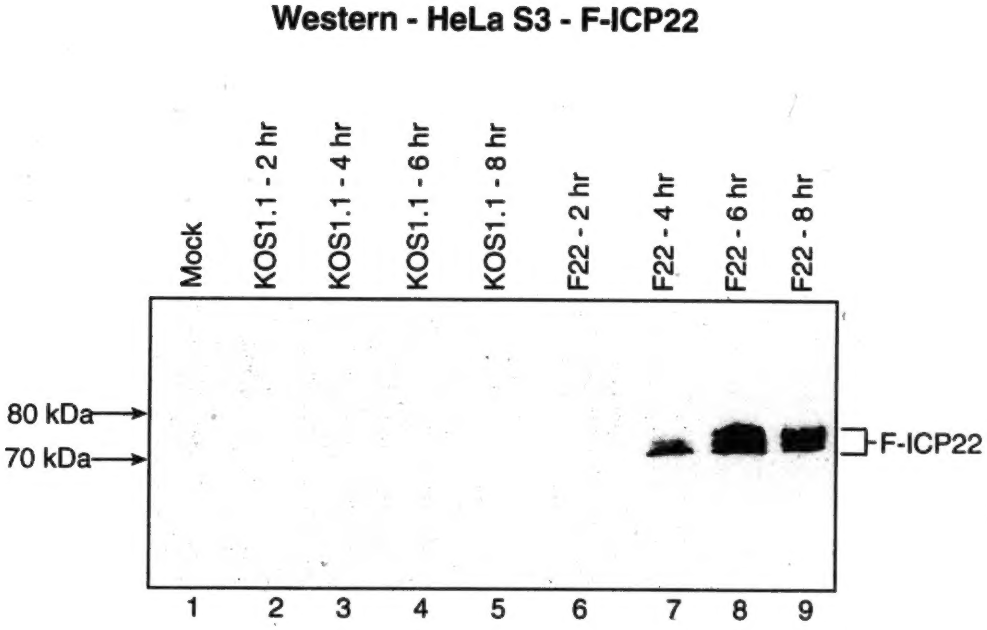

Western - HeLa S3 - F-ICP22
Mock
KOS1.1 - 2 hr
KOS1.1 - 4 hr
KOS1.1 - 6 hr
KOS1.1 - 8 hr
F22 - 2 hr
F22 - 4 hr
F22 - 6 hr
F22 - 8 hr
80 kDa
70 kDa
F-ICP22
1
2
3
4
5
6
7
8
9

Figure 9.6. Western blot analysis of F-ICP22 in F22 infected HeLa S3 cells. HeLa S3 cells were mock infected (lane 1) or infected with WT HSV-1 (KOS1.1) (lanes 2 to 5) or the HSV-1 recombinant FLAG ICP22 virus (F22) (lanes 6 to 9) for various times indicated. Cells were harvested and lysed into Laemmli buffer. Total cell protein was separated on SDS-PAGE (12% acrylamide) gels, immunoblotted and probed with the monoclonal antibody M2, which recognizes the 8 amino acid FLAG epitope. F-ICP22 migrates between 70 and 80 kDa.

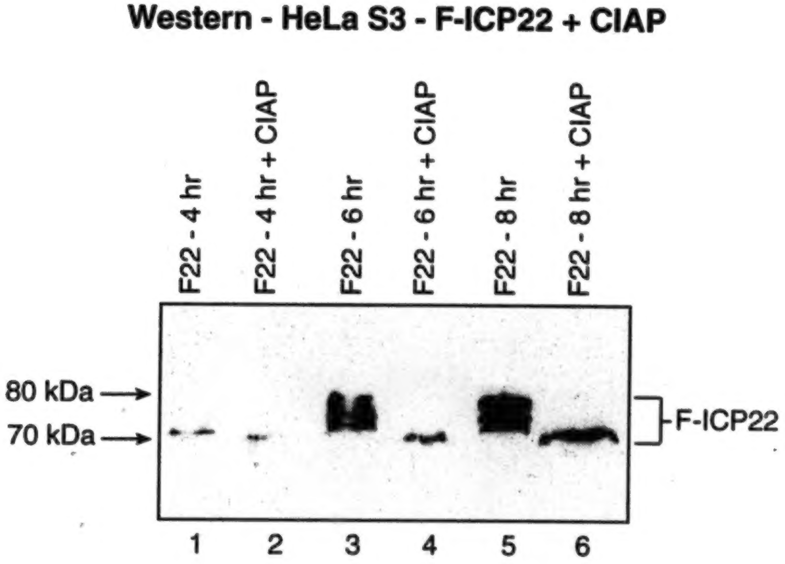

Western - HeLa S3 - F-ICP22 + CIAP
F22 - 4 hr
F22 - 4 hr + CIAP
F22 - 6 hr
F22 - 6 hr + CIAP
F22 - 8 hr
F22 - 8 hr + CIAP
80 kDa
70 kDa
F-ICP22
1
2
3
4
5
6

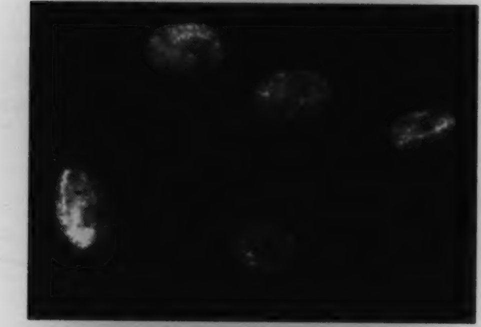
Figure 9.7. Western blot analysis of CIAP treated F-ICP22. Laemmli total cell extracts were prepared from mock infected HeLa S3 cells infected with the HSV-1 recombinant FLAG ICP22 virus (F22) for the times indicated. Whole cell extracts were treated with CIAP, separated by SDS-PAGE (12% acrylamide) and immunoblotted. The samples were probed with the M2 antibody, which recognizes the FLAG epitope of F-ICP22. Lanes: 1, 3, and 5, extracts untreated with CIAP; 2, 4, and 6, extracts treated with CIAP.

Figure 9.8. Nuclear localization of F-ICP22 in HSV-1 recombinant virus FLAG ICP22 virus (F22). Vero cells were infected with the HSV-1 recombinant virus FLAG ICP22 virus (F22) for 5 hr, after which the cells were fixed and stained with the anti-FLAG monoclonal antibody M2 and anti-ICP8 polyclonal antiserum 3-83. Secondary antibodies were a mixture of rhodamine-conjugated goat anti-mouse immunoglobulin (for F-ICP22 visualization) and fluorescein conjugated donkey anti-rabbit immunoglobulin (for ICP8 visualization). Phase-contrast microscopy (panel A). Detection of F-ICP22 (panel B). Detection of ICP8 (panel C).

Double Immunfluorescence

(A) F22 - phase

(B) F22 - F-ICP22



(C) F22 - ICP8

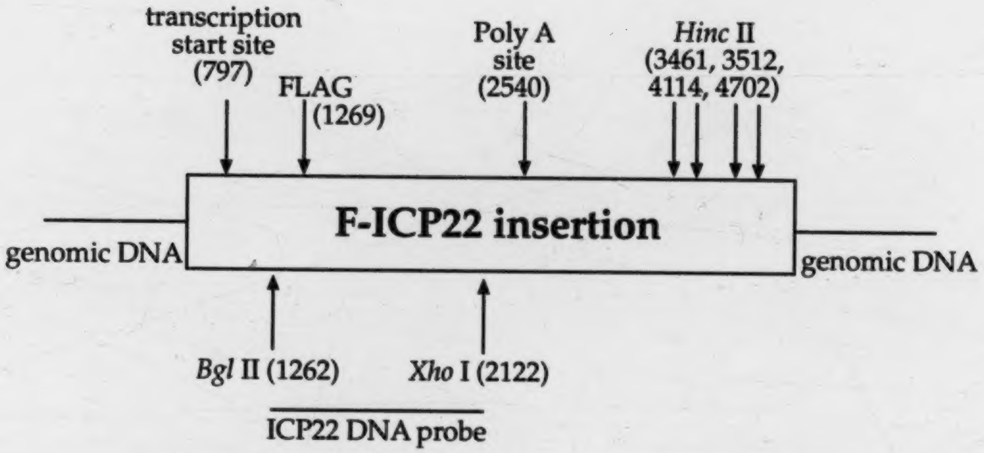

transcription start site (797)
FLAG (1269)
Poly A site (2540)
Hinc II (3461, 3512, 4114, 4702)
F-ICP22 insertion
genomic DNA
genomic DNA
Bgl II (1262)
Xho I (2122)
ICP22 DNA probe

Figure 9.9. Diagram of the F-ICP22 insertion in HeLa S3 genomic DNA. The box depicts the F-ICP22 insertion stably transfected into HeLa S3 genomic DNA, which is represented by the thin lines. The F-ICP22 gene contains the ICP22 transcription start site, FLAG sequences and the ICP22 poly (A) signal. Various restriction enzyme cleavage sites present in the gene are also shown. The ICP22 DNA probe (*Bgl* II-*Xho* I fragment) used in Southern and Northern blotting is indicated. The numbers represent the ICP22 DNA sequence.

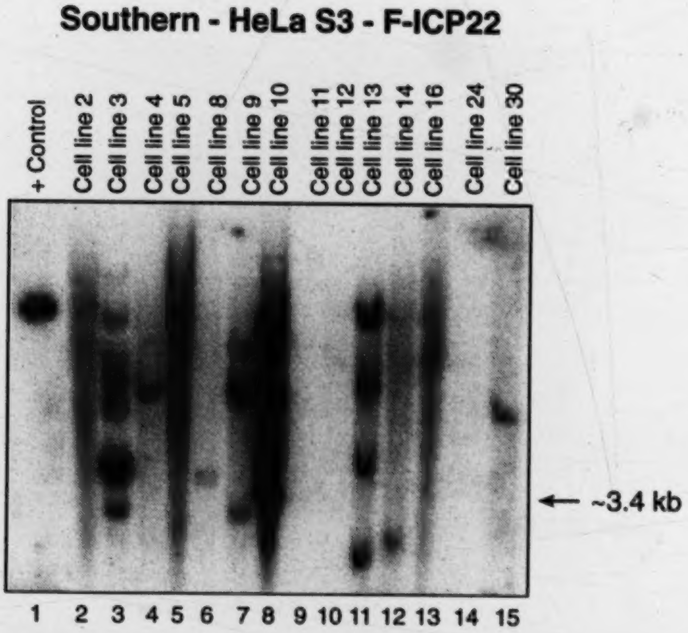

Southern - HeLa S3 - F-ICP22
+ Control
Cell line 2
Cell line 3
Cell line 4
Cell line 5
Cell line 8
Cell line 9
Cell line 10
Cell line 11
Cell line 12
Cell line 13
Cell line 14
Cell line 16
Cell line 24
Cell line 30
~3.4 kb
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 9.10. Southern blot analysis of stably transfected HeLa S3 F-ICP22 cell lines. The DNA from stably transfected HeLa S3 F-ICP22 cell lines, containing the F-ICP22 insertion, was isolated, as described in Chapter 2, and digested with the restriction enzyme *Hinc* II. DNA was separated on a 1% agarose gel and transferred by Southern blotting. The blot was probed with the radiolabeled ICP22 DNA probe as shown in Fig. 9.9. Lanes: 1, a positive control consisting of a 7.6 kb linearized DNA fragment containing the F-ICP22 gene; 2 to 15, DNA from HeLa S3 F-ICP22 cell lines. The minimal size of the F-ICP22 insertion when the DNA is digested with *Hinc* II is approximately 3.4 kb.

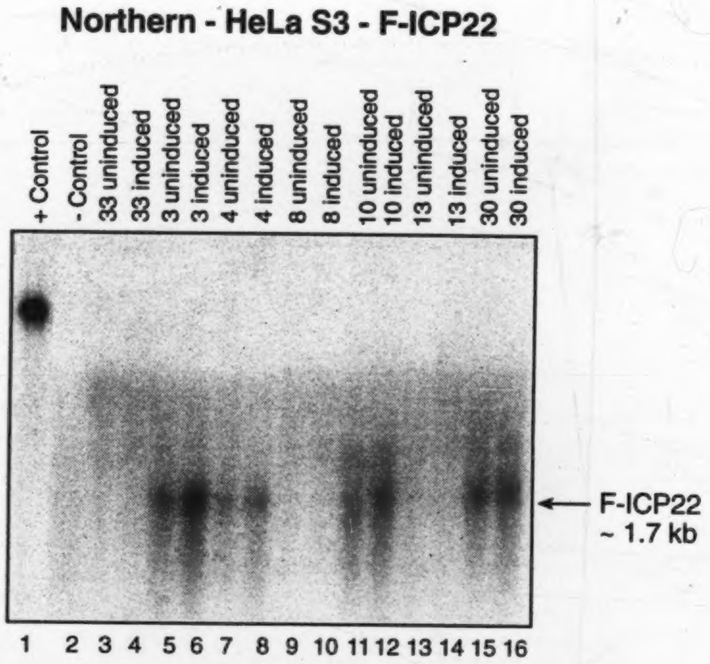
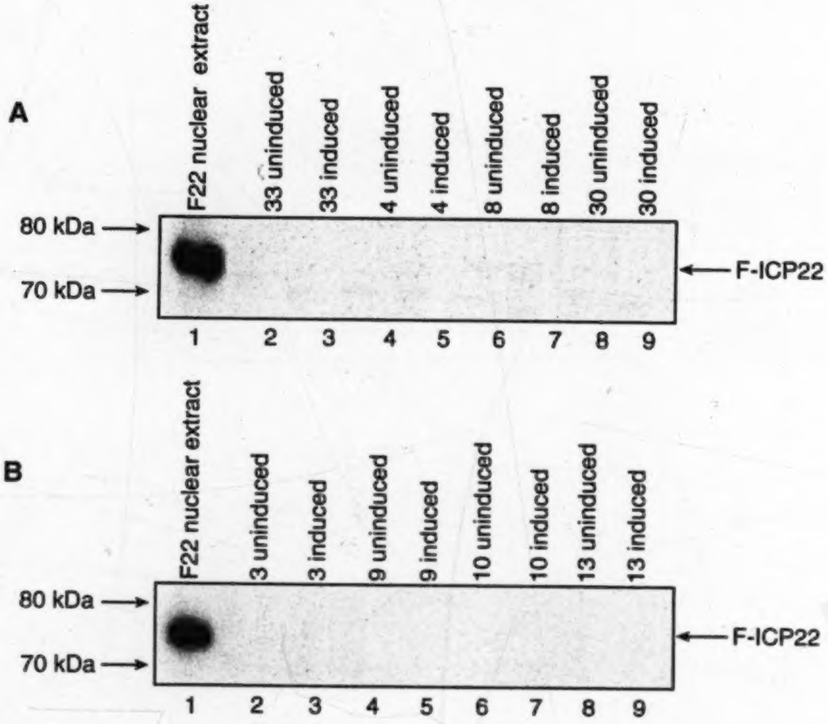

Northern - HeLa S3 - F-ICP22
+ Control
- Control
33 uninduced
33 induced
3 uninduced
3 induced
4 uninduced
4 induced
8 uninduced
8 induced
10 uninduced
10 induced
13 uninduced
13 induced
30 uninduced
30 induced
F-ICP22
~ 1.7 kb
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 9.11. Northern blot analysis of stably transfected HeLa S3 F-ICP22 cell lines. Stably transfected HeLa S3 F-ICP22 cell lines (3, 4, 8, 10, 13 and 30) containing the F-ICP22 gene were induced for F-ICP22 mRNA expression in the absence of tetracycline for 24 hr. Total RNA was harvested from uninduced (lanes 3, 5, 7, 9, 11, 13, and 15) and induced (lanes 4, 6, 8, 10, 12, 14, and 16) cell lines and run on an agarose (0.7%) formaldehyde gel. The samples were transferred by Northern blotting and probed with the radiolabeled ICP22 DNA probe as shown in Fig. 9.9. Lanes: 1, a positive control consisting of a 7.6 kb linearized DNA fragment containing the F-ICP22 gene; 2, a negative control consisting of total RNA harvested from nontransfected HeLa S3 cells; 3 and 4, RNA from uninduced and induced stably transfected HeLa S3 cell line 33, which expresses tet-VP16; 5 to 16, RNA from uninduced and induced HeLa S3 F-ICP22 cell lines. The F-ICP22 mRNA migrates at approximately 1.7 kb.

Figure 9.12. Western blot analysis of F-ICP22 in stably transfected HeLa S3 F-ICP22 cell lines. Stably transfected HeLa S3 F-ICP22 cell lines (3, 4, 8, 9, 10, 13, and 30) which have the F-ICP22 gene incorporated into the HeLa S3 genome were induced for F-ICP22 expression in the absence of tetracycline for 24 hr. Uninduced (panels A and B, lanes 2, 4, 6, and 8) and induced (panels A and B, lanes 3, 5, 7, and 9) cells were harvested and lysed into Laemmli buffer. The samples were separated on SDS-PAGE (12% acrylamide) gels and subjected to immunoblotting. Lane 1 is of a nuclear *in vitro* transcription extract prepared from HeLa S3 cells infected with the HSV-1 recombinant virus containing the F-ICP22 gene (F22). Panel A, lanes 2 and 3, are negative control extracts of uninduced and induced stably transfected HeLa S3 cell line 33, which expresses tet-VP16. The blot was probed with the M2 antibody, which recognizes the FLAG epitope of F-ICP22. F-ICP22 migrates between 70 and 80 kDa.

Western - HeLa S3 - F-ICP22


A
F22 nuclear extract
33 uninduced
33 induced
4 uninduced
4 induced
8 uninduced
8 induced
30 uninduced
30 induced
80 kDa
70 kDa
F-ICP22
1
2
3
4
5
6
7
8
9
B
F22 nuclear extract
3 uninduced
3 induced
9 uninduced
9 induced
10 uninduced
10 induced
13 uninduced
13 induced
80 kDa
70 kDa
F-ICP22
1
2
3
4
5
6
7
8
9

CHAPTER TEN. FLAG ICP22 (F-ICP22) AND ICP4 IMMUNOPRECIPITATIONS

A. Introduction

There is evidence in the literature to suggest that ICP22 function is mediated by interactions with other proteins. *In vitro*, ICP22 interacts with the Epstein-Barr virus small RNAs (EBER)-associated protein (EAP) (Leopardi et al., 1997), a host nucleolar protein that associates with ribosomes (Toczyski et al., 1994). EAP also binds to small RNAs of unknown function in Epstein-Barr virus infected cells (Toczyski and Steitz, 1991; Toczyski and Steitz, 1993). EAP interaction with ICP22 is independent of ICP4 (Leopardi et al., 1997), which also interacts with EAP (Leopardi and Roizman, 1996). The function of EAP during HSV-1 infection is currently unknown but it is possible that ICP22 and ICP4 modulate EAP activity. To date, EAP has no known transcriptional activity, but the protein may facilitate a functional interaction between ICP4 and ICP22 with each other or with other cellular factors (Leopardi et al., 1997). Binding of EAP to ICP4 decreases ICP4 binding to its cognate DNA sequence (Leopardi and Roizman, 1996).

The equine herpesvirus (EHV) homologue of HSV-1 ICP22 (EICP22) improves the *in vitro* binding activity of the EHV homologue of HSV-1 ICP4 (EICP4). It is thought that this may be facilitated by interactions between EICP4 and EICP22 (Kim et al., 1997b). Because EICP22 and EICP4 proteins share extensive homology to their HSV-1 counterparts (McGeoch et al., 1985; McGeoch and Davison, 1986; Grundy et al., 1989), it is possible that ICP22 also improves ICP4's DNA binding by direct interactions between HSV-1 ICP22 and HSV-1 ICP4.

In this chapter, I report the interactions of F-ICP22 with other IE and cellular proteins. Identifying the proteins that interact with F-ICP22 may help to delineate the biochemical pathway by which ICP22 functions and ultimately, provide a better understanding of how HSV-1 transcription regulation is mediated.

B. FLAG ICP22 (F-ICP22) immunoprecipitations

To determine if F-ICP22 interacts with other IE and cellular proteins, I performed coimmunoprecipitation assays. Nuclear extracts were prepared from uninfected, WT HSV-1 (KOS1.1), ICP4 mutant (*d*120), or F22 infected HeLa S3 cells. In addition, whole cell extracts were prepared from mock, KOS1.1, *d*120, or F22 infected Vero cells. These nuclear and cellular extracts were then immunoprecipitated with the M2 antibody, which specifically recognizes the 8 amino acid FLAG TAG of F-ICP22. The extracts were precleared and the Omnisorb cells were blocked with 4% BSA prior to immunoprecipitation. The immunoprecipitates were analyzed by SDS-PAGE and Western blotting with the M2 antibody (Fig. 10.1B).

Only F22 infected HeLa S3 nuclear extracts and F22 infected Vero cellular extracts contained F-ICP22 protein (Fig. 10.1A, lanes 4 and 8), whereas uninfected nuclear extracts and mock infected cellular extracts did not contain F-ICP22 protein (Fig. 10.1A, lanes 1 and 5). Moreover, F-ICP22 was not present in KOS1.1 and *d*120 infected nuclear and cellular extracts (Fig. 10.1A, lanes 2, 3, 6, and 7). Multiple forms of F-ICP22 were observed in F22 infected extracts and likely represent phosphorylation variants of the protein (Fig. 10.1A, lanes 4 and 8). F-ICP22 is extensively modified by phosphorylation after infection (Chapter 9; Purves and Roizman, 1992; Purves et al., 1993). F-ICP22 was efficiently immunoprecipitated with the M2 antibody from F22 infected HeLa S3 nuclear and Vero cellular extracts (Fig. 10.1B, lanes 6 and 10). It is unknown which phosphorylated forms of F-ICP22 are immunoprecipitated.

A series of controls were performed to ensure that the immunoprecipitation of F-ICP22 in F22 infected extracts was specific. Omnisorb cells did not contain any cross-reacting proteins that were nonspecifically recognized by the M2 antibody (Fig. 10.1B, lane 1). The immunoprecipitation of F-ICP22 was dependent on the M2 antibody, because F-ICP22 was not precipitated from F22 infected HeLa S3 nuclear extracts by the sole presence of Omnisorb cells (Fig. 10.1B, lane 2). The Omnisorb cells were also not nonspecifically precipitating F-ICP22 from F22 infected Vero cellular extracts (data not shown). These experiments show that the immunoprecipitation of F-ICP22 by the M2 antibody is specific.

To examine all proteins which coimmunoprecipitated with F-ICP22, I performed immunoprecipitations of F-ICP22 with ^{35}S-labeled Vero infected cellular extracts. Vero cells were mock, WT HSV-1 (KOS1.1), or F22 infected for two hours and then labeled with ^{35}S-methionine/cysteine. At 5 hr post-infection, the cells were harvested and the lysates were immunoprecipitated with the M2 antibody. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography (Fig. 10.2B).

The protein expression pattern in WT HSV-1 (KOS1.1) or F22 infected cells (Fig. 10.2A, lanes 2 and 3) differed from the pattern observed in mock infected cells (Fig. 10.2A, lane 1), likely due to the shift from cellular to viral transcription and translation after infection, as observed previously (Rice et al., 1993). Specific proteins, including ICP4, ICP5, ICP8, and actin were identified by their molecular weights in KOS1.1 and F22 infected cells. Proteins migrating between 70 to 80 kDa were immunoprecipitated with the M2 antibody in F22 infected cells (Fig. 10.2B, lane 4). This broad band likely represents multiple forms of F-ICP22 that are differentially phosphorylated. The immunoprecipitation of F-ICP22 by the M2 antibody appeared to be specific since a similar protein was not present in M2 immunoprecipitates of mock or KOS1.1 infected extracts (Fig. 10.2B, lanes 2 and 3).

Other proteins coimmunoprecipitated with F-ICP22. However, these proteins may be nonspecifically interacting with the Omnisorb cells because F22 infected cellular extracts incubated with Omnisorb cells, in the absence of the M2 antibody, resulted in the precipitation of these proteins (Fig. 10.2B, lane 1). Other proteins which may specifically form an immunocomplex with F-ICP22 were not readily identified in M2 immunoprecipitates of F22 infected cells. It is possible that nonspecific proteins precipitated by Omnisorb cells were obscuring the visualization of specific proteins that coimmunoprecipitated with F-ICP22. In addition, F-ICP22 interacting proteins may not be easily detected by these methods.

Because equine herpes virus (EHV) homologues of HSV-1 ICP22 and HSV-1 ICP4 may interact (Kim et al., 1997b), it was of interest to determine if HSV-1 proteins, ICP22 and ICP4, interact. To determine if ICP4 coimmunoprecipitated with F-ICP22, I stripped the Western blots in Fig. 10.1 and probed with H1101, an antibody which specifically recognizes ICP4 (Fig. 10.3). In KOS1.1 and F22 infected HeLa S3 nuclear and Vero cellular extracts prior to immunoprecipitation, full-length ICP4 was present (Fig. 10.3A, lanes 2, 4, 6, 8), whereas in *d*120 infected extracts, truncated ICP4 was observed (Fig. 10.3A, lanes 3 and 7). The *d*120 virus encodes a severely truncated ICP4 protein, consisting of the first 171 residues of the 1298 residue protein and is of approximately 30 kDa in molecular weight (DeLuca et al., 1985; DeLuca and Schaffer, 1988).

Full-length and truncated ICP4 were present in M2 immunoprecipitates of F22 infected nuclear and cellular extracts, demonstrating that ICP4 and F-ICP22 coimmunoprecipitate (Fig. 10.3B, lanes 6 and 10). Truncated ICP4 was present in the M2 immunoprecipitates of F22 infected extracts, suggesting that the N terminus of ICP4 interacts with F-ICP22. The F-ICP22 interacting domain of ICP4 is also required for the transactivation function of ICP4 (DeLuca and Schaffer, 1988; Xiao et al., 1997). ICP4 is post-translationally modified by phosphorylation (Pereira et al., 1977; Wilcox et al., 1980), poly-ADP-ribosylation *in vitro* (Preston and Notarianni, 1983; Blaho et al., 1993). We are uncertain as to the modifications present in coimmunoprecipitated ICP4. However, truncated ICP4 is thought to be nonphosphorylated, as it is not labeled with ^{32}P (DeLuca and Schaffer, 1988).

Proteins from the Omnisorb cells were not nonspecifically immunoprecipitated by the M2 antibody and then recognized by the H1101 antibody during Western blotting (Fig. 10.3B, lane 1). Furthermore, ICP4 in F22 infected HeLa S3 nuclear and Vero cellular extracts was not nonspecifically precipitated by the Omnisorb cells in the absence of the M2 antibody (Fig. 10.3B, lane 2, and data not shown). Full-length ICP4 and truncated ICP4 were not present in the M2 immunoprecipitates from KOS1.1 and *d*120 infected extracts respectively (Fig. 10.3B, lanes 4, 5, 8, and 9), indicating that the coimmunoprecipitation of ICP4 was dependent on the immunoprecipitation of F-ICP22 (Fig. 10.3B, lanes 6 and 10). These results show that the coimmunoprecipitation of ICP4 and F-ICP22 is specific.

I was also interested in determining if other IE proteins coimmunoprecipitated with F-ICP22. The Western blots in Fig. 10.3 were stripped and reprobed with antibodies specific for ICP0 and ICP27 (data not shown). I was unable to detect ICP0 and ICP27 in the F-ICP22 immunoprecipitates. I was also unable to determine conclusively if UL13, the viral kinase that is required for WT patterns of ICP22 phosphorylation (Purves and Roizman, 1992; Purves et al., 1993), coimmunoprecipitated with F-ICP22. The polyclonal UL13 antibody, R90/7 (Overton et al., 1992), was nonspecific and recognized many proteins in the M2 immunoprecipitates (data not shown).

To test whether cellular transcription proteins coimmunoprecipitated with F-ICP22, the Western blots in Fig. 10.3 were stripped and reprobed with antibodies specific for TBP and the large subunit of RNAP II (data not shown). We were unable to detect TBP and RNAP II large subunit in the M2 immunoprecipitates.

C. ICP4 immunoprecipitations

Since ICP4 is detected in F-ICP22 immunoprecipitates, we were interested in determining if F-ICP22 was detected in ICP4 immunoprecipitates. Nuclear extracts prepared from uninfected, WT HSV-1 (KOS1.1), ICP4 mutant (*d*120), or F22 infected HeLa S3 cells and cellular extracts prepared from mock, KOS1.1, *d*120, or F22 infected Vero cells were immunoprecipitated with H1101 or H1114, mouse monoclonal antibodies that are specifically directed against ICP4. H1101 recognizes the N-terminus of ICP4, between amino acids 1 and 171 (DeLuca and Schaffer, 1988), whereas H1114 recognizes regions of ICP4 following amino acid 171 (Leong [Lam] and Mears, 1996, Personal communication). Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with H1101 (Fig. 10.4B) and H1114 (Fig. 10.4B).

Full-length ICP4 was present in KOS1.1 and F22 infected HeLa S3 nuclear and Vero cellular extracts (Fig. 10.4A, lanes 2, 3, 7, and 8), whereas truncated ICP4 was present in *d*120 infected extracts (Fig. 10.4A, lanes 4 and 6). Full-length ICP4 was immunoprecipitated with H1101 in F22 and KOS1.1 infected HeLa S3 nuclear and Vero cellular extracts (Fig. 10.4B, lanes 4, 6, 8, and 10). Truncated ICP4 was also detected in the H1101 immunoprecipitates of *d*120 infected lysates (Fig. 10.4B, lanes 5 and 9).

Full-length ICP4 was present in KOS1.1 or F22 infected HeLa nuclear and Vero cellular extracts (Fig. 10.5A, lanes 2, 4, 5, and 8) and was immunoprecipitated with the H1114 antibody (Fig. 10.5B, lanes 4, 6, 8 and 10). As expected, ICP4 was not present in uninfected HeLa S3 nuclear and mock infected Vero cellular extracts (Fig. 10.5A, lanes 1 and 6). The truncated ICP4 protein produced by *d*120 infected cells was not detected with H1114, which does not recognize the first 171 residues of ICP4 (data not shown).

We are uncertain as to the post-translational modifications present in ICP4 in the virus infected lysates and in the H1101 and H1114 immunoprecipitates. ICP4 immunoprecipitated by H1114 appears to migrate as a doublet on SDS-PAGE gels (Fig. 10.5B, lanes 4, 6, 8, and 10), but it is unknown whether this doublet represents differently phosphorylated forms of ICP4 or whether the faster migrating band is proteolytically cleaved ICP4.

Control experiments were also performed to ensure that the immunoprecipitation of ICP4 in KOS1.1 and F22 infected extracts was specific. Proteins from Pansorbin cells did not precipitate with the ICP4 antibodies (Fig. 10.4B and 10.5B, lane 1). Pansorbin cells did not nonspecifically precipitate ICP4 from nuclear extracts prepared from F22 infected HeLa S3 cells (Fig. 10.4B and 10.5B, lane 2). Also, Pansorbin cells did not nonspecifically precipitate ICP4 from KOS1.1 infected HeLa S3 nuclear extracts or KOS1.1 or F22 infected Vero cellular extracts in the absence of ICP4 antibody (data not shown). These experiments demonstrate that immunoprecipitation of ICP4 by H1101 or H1114 is specific.

To determine whether F-ICP22 coimmunoprecipitated with ICP4 when immunoprecipitation of ICP4 was performed with H1101, we stripped the Western blots in Fig. 10.4 and reprobed with the M2 antibody (Fig 10.6). F-ICP22 was present in F22 infected HeLa S3 nuclear and Vero cellular extracts (Fig. 10.6A, lanes 3 and 8), but not in H1101 immunoprecipitates from these extracts, indicating that F-ICP22 and ICP4 did not coimmunoprecipitate (Fig. 10.6B, lanes 6 and 10). These results appear to contradict what was observed earlier, where ICP4 is found in M2 immunoprecipitates (Fig. 10.3B, lanes 6 and 10). The immunoprecipitation of ICP4 with H1101 may disrupt the interaction of ICP4 and F-ICP22. Alternatively, H1101 is unable to immunoprecipitate ICP4 that is complexed with F-ICP22. These are possibilities if the N-terminus of ICP4 that is recognized by H1101 is important for interactions with F-ICP22.

We then set out to determine if F-ICP22 coimmunoprecipitated with ICP4 when ICP4 was immunoprecipitated with H1114. The Western blots in Fig. 10.5 were stripped and reprobed with the M2 antibody (Fig. 10.7). F-ICP22 was detected in F22 infected HeLa S3 nuclear extracts and in F22 infected Vero cellular extracts (Fig. 10.7A, lanes 4 and 8) but not in uninfected HeLa S3 nuclear or mock infected Vero cellular extracts (Fig. 10.7A, lanes 1 and 6). F-ICP22 was also not present in KOS1.1 or *d*120 infected nuclear and cellular extracts (Fig. 10.7A, lanes 2, 3, 5, and 7). F-ICP22 was detectable in H1114 immunoprecipitates of F22 infected Vero cellular extracts (Fig. 10.7B, lane 10), but not in the immunoprecipitates of F22 infected HeLa S3 nuclear extracts (Fig. 10.7B, lane 6).

Proteins from Pansorbin cells were not nonspecifically immunoprecipitated by the H1114 antibody and then detected by the M2 antibody by Western blotting (Fig. 10.7B, lane 1). In addition, the Pansorbin cells were not nonspecifically precipitating F-ICP22 in F22 infected HeLa S3 nuclear (Fig. 10.7B, lane 2) or F22 infected Vero cellular extracts (data not shown). These results indicate that ICP4 specifically coimmunoprecipitates with F-ICP22.

Because coimmunoprecipitation of ICP4 with F- ICP22 was efficient with H1114 but not with H1101, this further supports the idea that interactions between these two proteins are mediated through the N-terminus of ICP4.

We have also performed immunoprecipitations of ICP4 with the H1114 antibody from ^{35}S-labeled Vero cellular lysates prepared after cycloheximide reversal of infected cells (data not shown). Vero cells were mock, KOS1.1 or F22 infected in the presence of cycloheximide. At 6 hr post-infection, the cycloheximide was washed out and the cells were labeled with ^{35}S-methionine/cysteine in the presence of actinomycin D. After 1 hr, the cells were harvested and the lysates were immunoprecipitated with H1114. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Using these methods, we found that ICP0 and ICP27 coimmunoprecipitated with ICP4, as observed previously (Yao and Schaffer, 1994; Panagiotidis et al., 1997). We were unable to conclusively determine whether F-ICP22 or WT ICP22 coimmunoprecipitated with ICP4. This may be a reflection of this particular experiment because the ^{35}S-labeling of ICP22 was much weaker than the other IE proteins and was not even detected in the lysates prior to immunoprecipitation (data not shown).

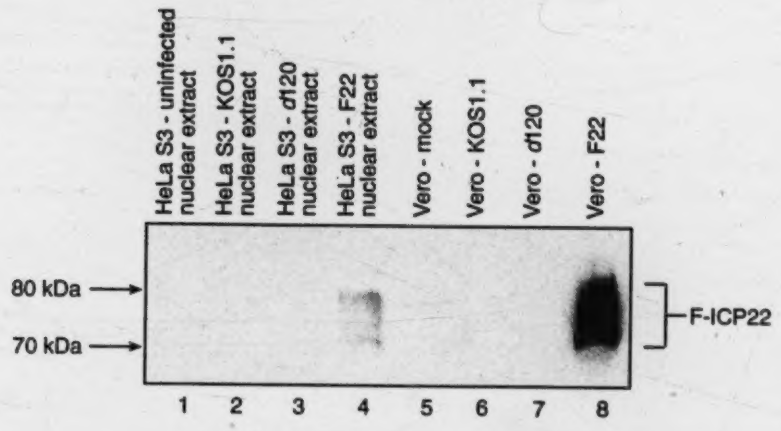
D. Summary

ICP4 is present in F-ICP22 immunoprecipitates and F-ICP22 is present in certain ICP4 immunoprecipitates. Both H1101 and H1114 antibodies are capable of immunoprecipitating ICP4, but F-ICP22 is only observed in H1114 immunoprecipitates, suggesting that the H1101 antibody may disrupt interactions of ICP4 and ICP22 that occur through the N-terminus 171 amino acids of ICP4. Alternatively, H1101 is unable to immunoprecipitate ICP4 that is complexed with F-ICP22. It is unknown whether the interaction between ICP4 and F-ICP22 is direct or facilitated by another protein. In addition, it is unknown which regions of F-ICP22 are required for coimmunoprecipitation with ICP4.

The interaction of ICP22 with ICP4 is intriguing, as we have noted that ICP22 is essential for efficient DE and L gene transcription (in some cell types) (Chapter 5; Rice et al., 1995), and ICP4 is the main transcriptional activator of DE and L genes (Preston, 1979; Watson and Clements, 1980; DeLuca et al., 1984; DeLuca and Schaffer, 1985; Godowski and Knipe, 1986). It is possible that ICP22's effects on viral gene transcription are mediated by its interactions with ICP4.

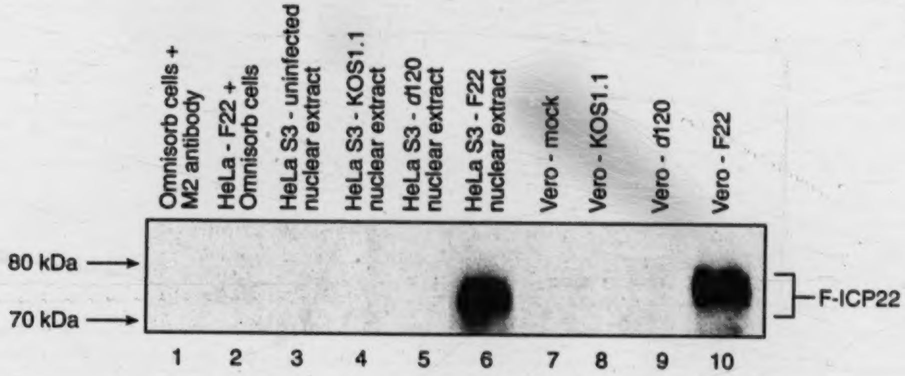
Interactions between F-ICP22 and other proteins *in vivo* may be transient and may not be easily observed by our methods. It is highly likely that there are other interactions between F-ICP22 and other IE and cellular proteins that occur but are not detected by our stringent immunoprecipitation methods. Moreover, F-ICP22 and ICP4 coimmunoprecipitation found under our experimental conditions but may not reflect the *in vivo* situation. In spite of this, our findings suggest that interactions between viral IE proteins may play a role in the regulatory functions of F-ICP22 during HSV-1 gene expression.

Figure 10.1. F-ICP22 is immunoprecipitated with the M2 antibody. (A) Western blot detection of F-ICP22 in nuclear and cellular extracts used for immunoprecipitation. HeLa S3 cells were uninfected (lane 1) or infected with WT HSV-1 (KOS1.1) (lane 2), ICP4 mutant (*d*120) (lane 3), or HSV-1 recombinant virus containing the F-ICP22 gene (F22) (lane 4) for 10 to 12 hr. Nuclear extracts were prepared as described in Chapter 2. In addition, Vero cells were mock (lane 5), KOS1.1 (lane 6), *d*120 (lane 7), or F22 (lane 8) infected for 5 to 6 hr. Cells were harvested and whole cell lysates were prepared. Nuclear and cellular extracts were run on SDS-PAGE (12% acrylamide) gels, immunoblotted and probed with the M2 antibody, which recognizes the FLAG epitope of F-ICP22. F-ICP22 migrates between approximately 70 to 80 kDa. (B) Western blot detection of immunoprecipitated F-ICP22. Nuclear (lanes 3 to 6) and cellular (lanes 7 to 10) extracts prepared as described above were immunoprecipitated with the M2 antibody, as described in Chapter 2. The immunoprecipitates were analyzed by Western blotting as described above. Lane 1 is a control immunoprecipitation containing only Omnisorb cells and the M2 antibody in the absence of extract. Lane 2 is a control precipitation of a F22 infected HeLa nuclear extract with Omnisorb cells in the absence of M2 antibody. The blot was probed with the M2 antibody.



(A) Western - Lysates - F-ICP22

HeLa S3 - uninfected nuclear extract
HeLa S3 - KOS1.1 nuclear extract
HeLa S3 - d120 nuclear extract
HeLa S3 - F22 nuclear extract
Vero - mock
Vero - KOS1.1
Vero - d120
Vero - F22
80 kDa
70 kDa
F-ICP22
1
2
3
4
5
6
7
8



(B) Western - M2 Immunoprecipitates - F-ICP22

Omnisorb cells + M2 antibody
HeLa - F22 + Omnisorb cells
HeLa S3 - uninfected nuclear extract
HeLa S3 - KOS1.1 nuclear extract
HeLa S3 - d120 nuclear extract
HeLa S3 - F22 nuclear extract
Vero - mock
Vero - KOS1.1
Vero - d120
Vero - F22
80 kDa
70 kDa
F-ICP22
1
2
3
4
5
6
7
8
9
10

Figure 10.2. M2 immunoprecipitation of ^{35}S-labeled Vero infected cell extracts. (A) Autoradiograph of ^{35}S labeled cell extracts. Vero cells were mock (lane 1), WT HSV-1 (KOS1.1) (lane 2), or F22 (lane 3) infected for 2 hr and then labeled with ^{35}S-methionine/cysteine. Between 5 and 6 hr post-infection, the cells were harvested, separated by SDS-PAGE (12% acrylamide), and exposed to film. (B) Prepared cell extracts from mock (lane 2), KOS1.1 (lane 3), and F22 (lane 4) infected cells as described above were immunoprecipitated with the M2 antibody, which recognizes the FLAG epitope of F-ICP22. The immunoprecipitates were analyzed as described above. Lane 1 is a control precipitation of an cell extract prepared from F22 infected Vero cells with Omnisorb cells in the absence of the M2 antibody. F-ICP22 runs approximately between 70 and 80 kDa.

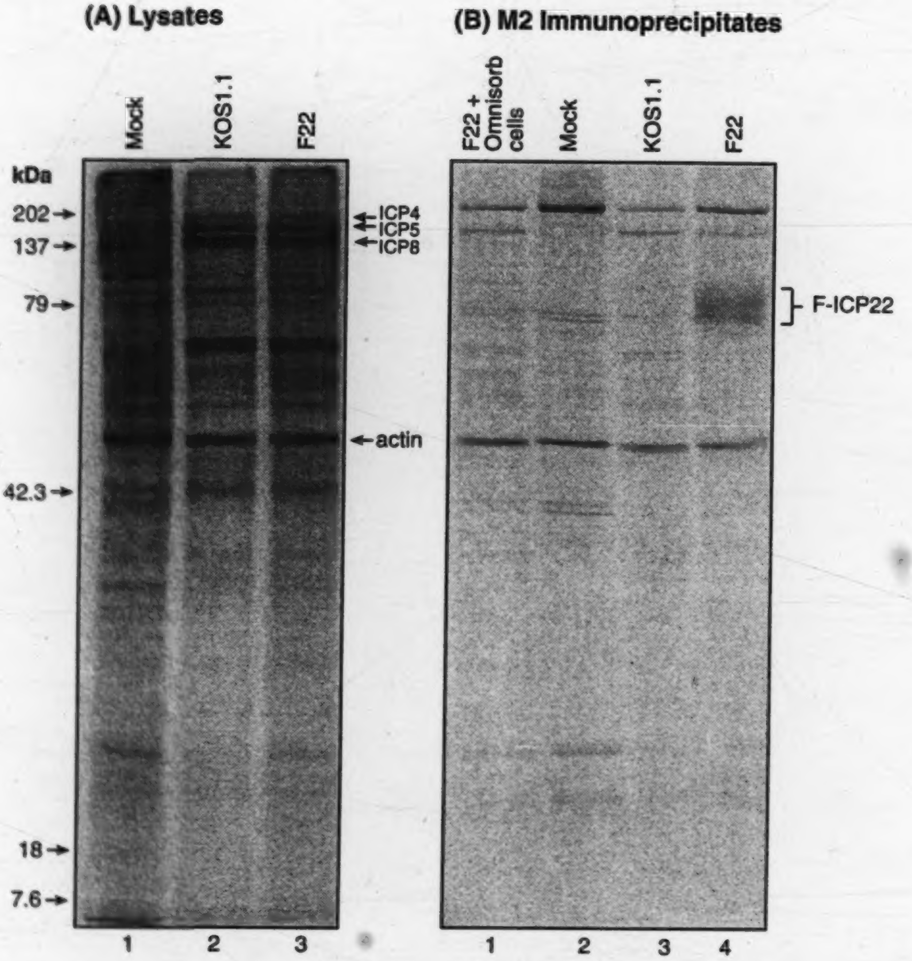

(A) Lysates
(B) M2 Immunoprecipitates
Mock
KOS1.1
F22
F22 + Omnisorb cells
Mock
KOS1.1
F22
kDa
202
137
79
42.3
18
7.6
ICP4
ICP5
ICP8
F-ICP22
actin
1
2
3
1
2
3
4

Figure 10.3. ICP4 is present in M2 immunoprecipitates. (A) Western blot detection of ICP4 in nuclear and cellular extracts used for immunoprecipitation. HeLa S3 cells were uninfected (lane 1) or infected with WT HSV-1 (KOS1.1) (lane 2), ICP4 mutant (*d*120) (lane 3), or HSV-1 recombinant virus containing the F-ICP22 gene (F22) (lane 4) for 10 to 12 hr. Nuclear extracts were prepared as described in Chapter 2. In addition, Vero cells were mock (lane 5), KOS1.1 (lane 6), *d*120 (lane 7), or F22 (lane 8) infected for 5 to 6 hr. Cells were harvested and lysates were prepared. Nuclear and cellular extracts were run on SDS-PAGE (12% acrylamide) gels, immunoblotted and probed with the H1101 antibody, which specifically recognizes ICP4. Full-length and truncated ICP4 migrates at approximately 175 kDa and 30 kDa respectively. (B) Western blot detection of immunoprecipitated ICP4. Nuclear (top and bottom panels, lanes 3 to 6) and cellular (top and bottom panels, lanes 7 to 10) extracts as described above were immunoprecipitated with the M2 antibody, as described in Chapter 2. The immunoprecipitates were analyzed by Western blotting as described above, except that the blot was probed with the H1101 antibody for ICP4. Lane 1 is a control immunoprecipitation containing only Omnisorb cells and M2 antibody in the absence of extract. Lane 2 is a control precipitation of a F22 infected HeLa nuclear extract with Omnisorb cells in the absence of M2 antibody. The top panel is exposed to film 5 to 10 times shorter than the bottom panel.

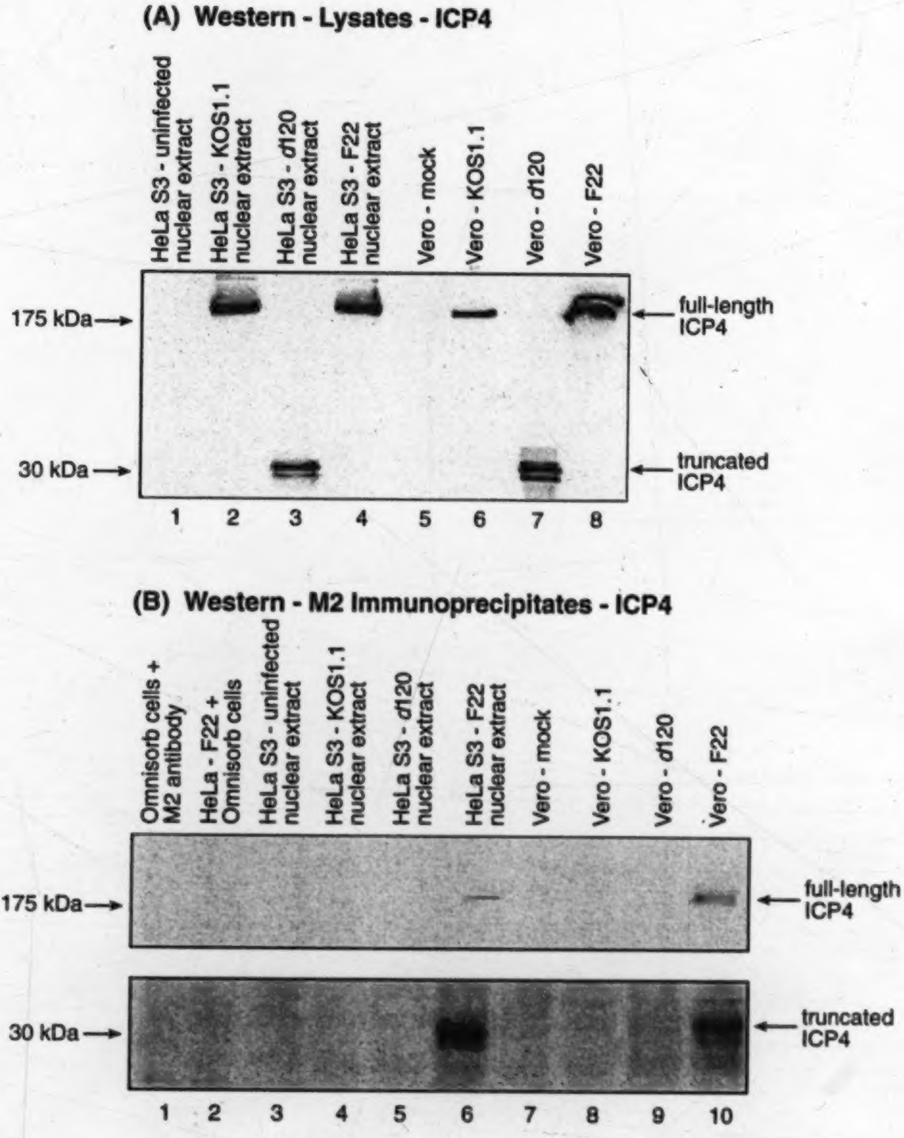
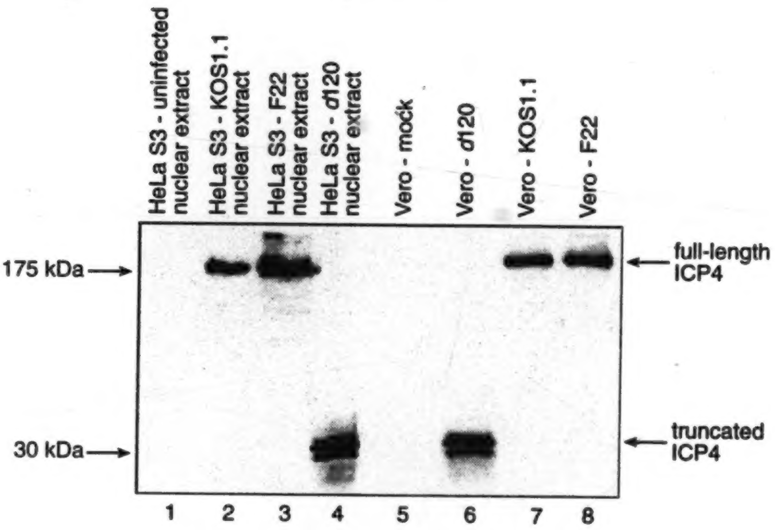
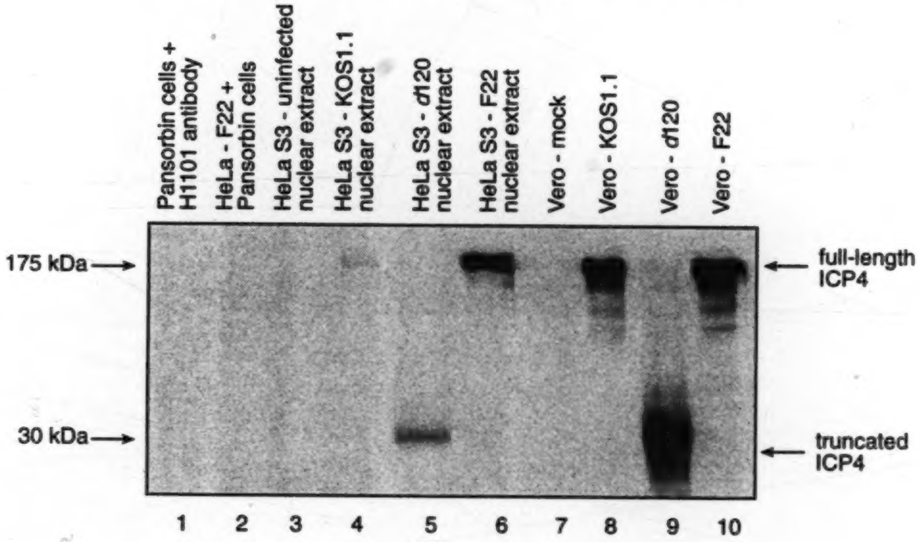

(A) Western - Lysates - ICP4
HeLa S3 - uninfected nuclear extract
HeLa S3 - KOS1.1 nuclear extract
HeLa S3 - d120 nuclear extract
HeLa S3 - F22 nuclear extract
Vero - mock
Vero - KOS1.1
Vero - d120
Vero - F22
175 kDa
30 kDa
full-length ICP4
truncated ICP4
1 2 3 4 5 6 7 8
(B) Western - M2 Immunoprecipitates - ICP4
Omnisorb cells + M2 antibody
HeLa - F22 + Omnisorb cells
HeLa S3 - uninfected nuclear extract
HeLa S3 - KOS1.1 nuclear extract
HeLa S3 - d120 nuclear extract
HeLa S3 - F22 nuclear extract
Vero - mock
Vero - KOS1.1
Vero - d120
Vero - F22
175 kDa
30 kDa
full-length ICP4
truncated ICP4
1 2 3 4 5 6 7 8 9 10

Figure 10.4. ICP4 is immunoprecipitated with the H1101 antibody. (A) Western blot detection of ICP4 in nuclear and cellular extracts used for immunoprecipitation. HeLa S3 cells were uninfected (lane 1) or infected with WT HSV-1 (KOS1.1) (lane 2), HSV-1 recombinant virus containing the F-ICP22 gene (F22) (lane 3), or ICP4 mutant (*d*120) (lane 4) for 10 to 12 hr. Nuclear extracts were prepared as described in Chapter 2. In addition, Vero cells were mock (lane 5), *d*120 (lane 6), KOS1.1 (lane 7), or F22 (lane 8) infected for 5 or 6 hr. Cells were harvested and extracts were prepared. Nuclear and cellular extracts were run on SDS-PAGE (12% acrylamide) gels, immunoblotted and probed with the H1101 antibody, which recognizes ICP4. Full-length and truncated ICP4 migrates at approximately 175 kDa and 30 kDa respectively. (B) Western blot detection of immunoprecipitated ICP4. Nuclear (lanes 3 to 6) and cellular (lanes 7 to 10) extracts as described above were immunoprecipitated with the H1101 antibody, as described in Chapter 2. The immunoprecipitates were analyzed by Western blotting as described above. Lane 1 is a control immunoprecipitation of Pansorbin cells and the H1101 antibody in the absence of extract. Lane 2 is a control precipitation of a F22 infected HeLa nuclear extract with Pansorbin cells in the absence of the H1101 antibody. The blot was probed for ICP4 using H1101.



(A) Western - Lysates - ICP4

HeLa S3 - uninfected nuclear extract
HeLa S3 - KOS1.1 nuclear extract
HeLa S3 - F22 nuclear extract
HeLa S3 - d120 nuclear extract
Vero - mock
Vero - d120
Vero - KOS1.1
Vero - F22
175 kDa
30 kDa
full-length ICP4
truncated ICP4
1
2
3
4
5
6
7
8

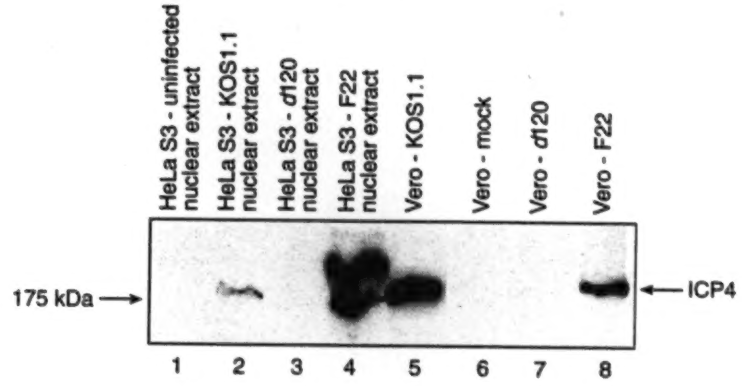


(B) Western - H1101 Immunoprecipitates - ICP4

Pansorbin cells + H1101 antibody
HeLa - F22 + Pansorbin cells
HeLa S3 - uninfected nuclear extract
HeLa S3 - KOS1.1 nuclear extract
HeLa S3 - d120 nuclear extract
HeLa S3 - F22 nuclear extract
Vero - mock
Vero - KOS1.1
Vero - d120
Vero - F22
175 kDa
30 kDa
full-length ICP4
truncated ICP4
1
2
3
4
5
6
7
8
9
10

Figure 10.5. ICP4 is immunoprecipitated with the H1114 antibody. (A) Western blot detection of ICP4 in nuclear and cellular extracts used for immunoprecipitation. HeLa S3 cells were uninfected (lane 1) or infected with WT HSV-1 (KOS1.1) (lane 2), ICP4 mutant (*d*120) (lane 3), or HSV-1 recombinant containing the F-ICP22 gene (F22) (lane 4) for 10 to 12 hr. Nuclear extracts were prepared as described in Chapter 2. In addition, Vero cells were KOS1.1 (lane 5), mock (lane 6), *d*120 (lane 7), or F22 (lane 8) infected for 5 or 6 hr. Cells were harvested and lysates were prepared. Nuclear and cellular extracts were run on SDS-PAGE (12% acrylamide) gels, immunoblotted and probed with the H1114 antibody, which recognizes ICP4. Full-length ICP4 migrates at approximately 175 kDa. The H1114 antibody does not recognize the 30 kDa truncated protein, suggesting that its epitope is towards the C terminus of the ICP4 protein. (B) Western blot detection of immunoprecipitated ICP4. Nuclear (lanes 3 to 6) and cellular (lanes 7 to 10) extracts as described above were immunoprecipitated with the H1114 antibody, as described in Chapter 2. The immunoprecipitates were analyzed by Western blotting as described above. Lane 1 is a control immunoprecipitation containing Pansorbin cells and the H1114 antibody in the absence of extract. Lane 2 is a control precipitation of a F22 infected HeLa nuclear extract with Pansorbin cells alone in the absence of the H1114 antibody. The blot was probed for ICP4 using H1114.

(A) Western - Lysates - ICP4


HeLa S3 - uninfected nuclear extract
HeLa S3 - KOS1.1 nuclear extract
HeLa S3 - d120 nuclear extract
HeLa S3 - F22 nuclear extract
Vero - KOS1.1
Vero - mock
Vero - d120
Vero - F22
175 kDa
ICP4
1
2
3
4
5
6
7
8

(B) Western - H1114 Immunoprecipitates - ICP4

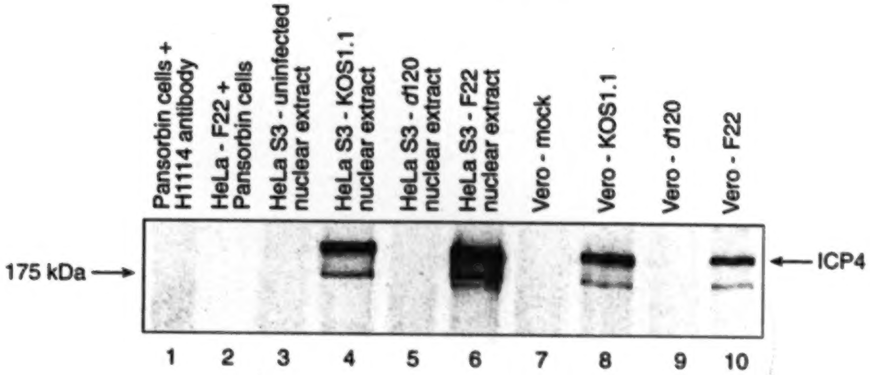
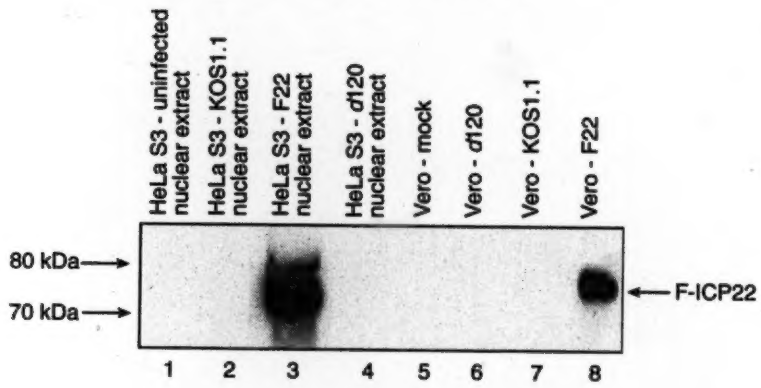

Pansorbin cells + H1114 antibody
HeLa - F22 + Pansorbin cells
HeLa S3 - uninfected nuclear extract
HeLa S3 - KOS1.1 nuclear extract
HeLa S3 - d120 nuclear extract
HeLa S3 - F22 nuclear extract
Vero - mock
Vero - KOS1.1
Vero - d120
Vero - F22
175 kDa
ICP4
1
2
3
4
5
6
7
8
9
10

Figure 10.6. F-ICP22 is not present in H1101 immunoprecipitates. (A) Western blot detection of F-ICP22 in nuclear and cellular extracts used for immunoprecipitation. HeLa S3 cells were uninfected (lane 1) or infected with WT HSV-1 (KOS1.1) (lane 2), HSV-1 recombinant virus containing the F-ICP22 gene (F22) (lane 3), or ICP4 mutant (*d*120) (lane 4) for 10 to 12 hr. Nuclear extracts were prepared as described in Chapter 2. In addition, Vero cells were mock (lane 5), *d*120 (lane 6), KOS1.1 (lane 7), or F22 (lane 8) infected for 5 to 6 hr. Cells were harvested and extracts were prepared. Nuclear and cellular extracts were run on SDS-PAGE (12% acrylamide) gels, immunoblotted and probed with the M2 antibody, which recognizes the FLAG epitope of F-ICP22. F-ICP22 migrates between 70 and 80 kDa. (B) Western blot detection of immunoprecipitated F-ICP22. Nuclear (lanes 3 to 6) and cellular (lanes 7 to 10) extracts as described above were immunoprecipitated with the H1101 antibody, as described in Chapter 2. The immunoprecipitates were analyzed by Western blotting as described above, except that the blot was probed for F-ICP22 using the M2 antibody. Lane 1 is a control immunoprecipitation containing Pansorbin cells and the H1101 antibody in the absence of extract. Lane 2 is a control precipitation of a F22 infected HeLa nuclear extract with Pansorbin cells alone in the absence of the H1101 antibody.

(A) Western - Lysates - F-ICP22


HeLa S3 - uninfected nuclear extract
HeLa S3 - KOS1.1 nuclear extract
HeLa S3 - F22 nuclear extract
HeLa S3 - dl120 nuclear extract
Vero - mock
Vero - dl120
Vero - KOS1.1
Vero - F22
80 kDa
70 kDa
F-ICP22
1
2
3
4
5
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7
8

(B) Western - H1101 Immunoprecipitates - F-ICP22

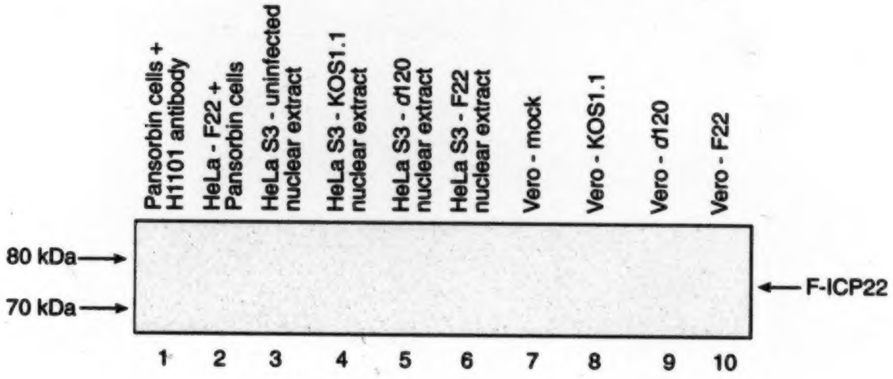
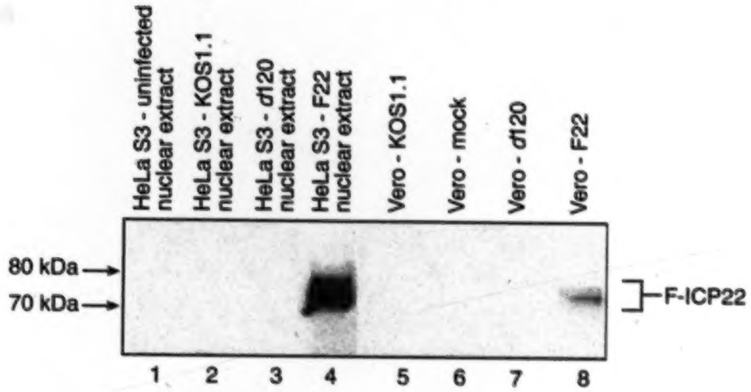
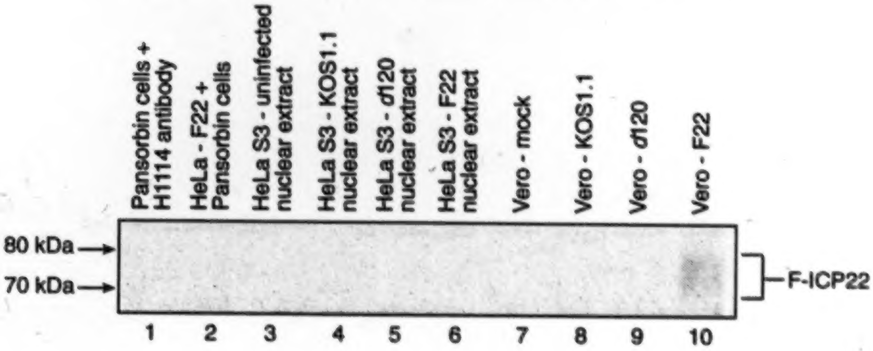

Pansorbin cells + H1101 antibody
HeLa - F22 + Pansorbin cells
HeLa S3 - uninfected nuclear extract
HeLa S3 - KOS1.1 nuclear extract
HeLa S3 - dl120 nuclear extract
HeLa S3 - F22 nuclear extract
Vero - mock
Vero - KOS1.1
Vero - dl120
Vero - F22
80 kDa
70 kDa
F-ICP22
1
2
3
4
5
6
7
8
9
10

Figure 10.7. F-ICP22 is present in H1114 immunoprecipitates. (A) Western blot detection of F-ICP22 in nuclear and cellular extracts used for immunoprecipitation. HeLa S3 cells were uninfected (lane 1) or infected with WT HSV-1 (KOS1.1) (lane 2), ICP4 mutant (*d*120) (lane 3), or HSV-1 recombinant virus containing the F-ICP22 gene (F22) (lane 4) for 10 to 12 hr. Nuclear extracts were prepared as described in Chapter 2. In addition, Vero cells were KOS1.1 (lane 5), mock (lane 6), *d*120 (lane 7), or F22 (lane 8) infected for 5 to 6 hr. Cells were harvested and lysates were prepared. Nuclear and cellular extracts were run on SDS-PAGE (12% acrylamide) gels, immunoblotted and probed with the M2 antibody, which recognizes the FLAG epitope of F-ICP22. F-ICP22 migrates between 70 and 80 kDa. (B) Western blot detection of immunoprecipitated F-ICP22. Nuclear (lanes 3 to 6) and cellular (lanes 7 to 10) extracts as described above were immunoprecipitated with the H1114 antibody, as described in Chapter 2. The immunoprecipitates were analyzed by Western blotting as described above, except the blot was probed with the M2 antibody. Lane 1 is a control immunoprecipitation containing Pansorbin cells and the H1114 antibody in the absence of extract. Lane 2 is a control precipitation of a F22 infected HeLa nuclear extract with Pansorbin cells alone in the absence of the H1114 antibody.

(A) Western - Lysates - F-ICP22


HeLa S3 - uninfected nuclear extract
HeLa S3 - KOS1.1 nuclear extract
HeLa S3 - *d*120 nuclear extract
HeLa S3 - F22 nuclear extract
Vero - KOS1.1
Vero - mock
Vero - *d*120
Vero - F22
80 kDa
70 kDa
F-ICP22
1
2
3
4
5
6
7
8

(B) Western - H1114 Immunoprecipitates - F-ICP22


Pansorbin cells + H1114 antibody
HeLa - F22 + Pansorbin cells
HeLa S3 - uninfected nuclear extract
HeLa S3 - KOS1.1 nuclear extract
HeLa S3 - *d*120 nuclear extract
HeLa S3 - F22 nuclear extract
Vero - mock
Vero - KOS1.1
Vero - *d*120
Vero - F22
80 kDa
70 kDa
F-ICP22
1
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5
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CHAPTER ELEVEN. DISCUSSION

A. Introduction

During HSV-1 infection, host transcription is repressed and viral transcription is activated. The expression of the viral genome is dependent on the host transcription machinery, which is redirected from cellular genes to viral genes. Viral gene expression is regulated primarily at the transcription level in a ordered temporal cascade and is tightly controlled by viral proteins that enter the cell as components of the virion or are synthesized early in infection (Roizman and Sears, 1990; Smiley et al., 1991).

The IE genes are transcriptionally activated by VP16 in association with cellular factors (McKnight et al., 1987; Gerster and Roeder, 1988; O'Hare and Goding, 1988; Preston et al., 1988; Triezenberg et al., 1988). However, the mechanisms by which viral DE and L genes are transcriptionally activated are unknown. DE and L gene promoters contain binding sites for cellular transcription factors, including Sp1 and TFIID (Coen et al., 1986; Johnson and Everett, 1986; McKnight and Tjian, 1986; Homa et al., 1988), but discrete *cis*-acting sequences which bind viral regulatory proteins and specifically activate transcription of the DE and L genes have not been identified.

The selective transcription of HSV-1 genes may not rely on differences in nucleotide sequences between viral and cellular promoters (Silver and Roizman, 1985; Smiley et al., 1991). Viral genes and their promoters, outside the context of the viral genome, are regulated differently from viral genes present in their natural context. In addition, cellular genes with their own promoters are expressed as viral DE genes when cloned into the viral genome. The endogenous host copies of these same genes are transcriptionally repressed after HSV-1 infection (Smiley et al., 1991). Therefore, the global shifts in transcription patterns that occur during HSV-1 infection may involve sequence-independent mechanisms such as the mobilization of cellular transcription factors, modification of the RNAP II transcription apparatus, differences in higher order chromatin packaging, or transport of transcription factors into specialized nuclear compartments (Smiley et al., 1991).

In this study, I have addressed the question of how HSV-1 brings about the global shifts in RNAP II transcription that occur following infection. My data suggest that virus-induced modifications to the RNAP II basal transcription apparatus may be involved in redirecting the transcription machinery from the cellular genome to the viral genome. The data also indicate that aberrant phosphorylation of the large subunit of RNAP II, as well as repression of host transcription and efficient activation of viral transcription, requires the expression of several viral IE proteins. This study is the first to examine modifications to the RNAP II transcription machinery following HSV-1 infection.

B. Modifications to RNAP II basal transcription machinery following HSV-1 infection

1. The large subunit of RNAP II is aberrantly phosphorylated following HSV-1 infection

We find that the large subunit of RNAP II is rapidly and aberrantly phosphorylated following WT HSV-1 infection. This modification involves the depletion of the IIa and IIo forms of the RNAP II large subunit, and the appearance of polydisperse intermediately-migrating forms, which we have termed IIi. Although IIa and IIo exist *in vivo* and *in vitro*, IIi is not generally detected in normal cells (Dahmus, 1994; Dahmus, 1996). Intermediately phosphorylated forms of the large subunit are detectable during phosphorylation of the large subunit *in vitro* (Kim and Dahmus, 1986; Cadena and Dahmus, 1987; Zhang and Corden, 1991a), but only in limiting concentrations of ATP (Dahmus, 1994).

RNAP II modifications during HSV-1 infection occur in more than one cell line, including Vero, HeLa S3, and HEL 299 cells. Although there are differences in the relative amounts of IIa and IIo in each cell line prior to infection, IIo and IIa are depleted and IIi is efficiently formed in all WT HSV-1 infected cells examined.

We have verified that IIi is a phosphorylation variant of the large subunit of RNAP II and is not produced by proteolysis of IIo. However, we cannot determine from our data the extent of IIi phosphorylation. The mobility shift of the large subunit of RNAP II on SDS-PAGE gels is not linear with respect to the number of phosphates present on the CTD. We suspect that fewer than 40 phosphates are incorporated into IIi. This prediction is based on the work of Payne and Dahmus (1993) who show that the addition of 40 phosphates to native calf thymus IIa is sufficient to shift the large subunit to the IIo position on SDS-PAGE gels. Based on the work of Zhang and Corden (1991b), we estimate that fewer than 10 phosphates are present on IIi. They noted a shift of recombinant CTD to the IIo position on SDS-PAGE gels after addition of approximately 7 to 9 phosphates per CTD *in vitro*. We cannot rule out the possibility that the IIi subunit contains a greater number of phosphates than the IIo form. Zhang and Corden (1991b) report that addition of extra phosphates to the IIo subunit decreases the mobility of recombinant CTD to a position below that of IIo on SDS-PAGE gels. However, the reactivity patterns of the 8WG16 antibody suggests that the CTD of IIi is hypophosphorylated. As observed in Western blots (Chapter 3 and 4), the 8WG16 antibody does not react with the hyperphosphorylated (IIo) forms of the large subunit; however, 8WG16 reacts strongly with both the nonphosphorylated (IIa) and the IIi form.

We have not conclusively determined whether phosphorylation of IIi is confined to the CTD. We have been unsuccessful in our attempts to cleave the CTD from the large subunit and measure incorporation of radioactive phosphate. It is clear that the IIo form of the large subunit is absent by 5 hr post-infection; therefore normal CTD phosphorylation is not occurring in infected cells. It is unlikely, but not impossible, that phosphorylation on the body of the large subunit would be sufficiently extensive to create the mobility shift that we observe between IIa and IIi. If such were the case, virus-induced modifications to the large subunit of RNAP II would be even more remarkable, involving both the loss of CTD phosphorylation and dramatic increases in phosphorylation on the body of the large subunit.

Phosphorylation of the CTD consensus sequence YSPTSPS (Corden et al., 1985) occurs on serine, threonine and tyrosine residues (Corden, 1990;

Corden and Ingles, 1992; Baskaran et al., 1993). Serines are the predominant sites of phosphorylation (Buhler et al., 1976; Cadena and Dahmus, 1987; Zhang and Corden, 1991a). Our phosphoamino acid analyses show that both IIo and IIi subunits are phosphorylated on serine and threonine residues. Furthermore, the ratio of serine to threonine phosphorylation is very similar in both forms. Although these data show little change in the ratio of serine to threonine phosphorylation after HSV-1 infection, they do not address the question of total numbers of phosphates or the specific sites of phosphorylation in the IIo and IIi forms of the large subunit.

Although it has been reported that the CTD of IIo is phosphorylated on tyrosine residues (Baskaran et al., 1993), we do not detect phosphotyrosines in either IIo or IIi subunits. This may be due to several reasons. Tyrosine dephosphorylation may be occurring during our nuclear lysate preparations. In addition, tyrosine phosphorylation of RNAP II may be cell type specific. Baskaran et al. (1993) examined the phosphoamino acid composition of IIo recovered from HeLa S3 cells, whereas we analyzed IIo and IIi from Vero cells. HeLa S3 and Vero cells may contain different levels of CTD tyrosine kinase or phosphatase activities. Lastly, phosphotyrosines in IIo and IIi may not be observed in our experiments because phosphotyrosines are very labile and difficult to detect in phosphoamino acid analyses.

Besides being phosphorylated, the RNAP II large subunit is also glycosylated (*O*-linked) on serine and threonine residues (Kelly et al., 1993; Dahmus, 1994). Although the function of *O*-linked glycosylation is unknown it has been suggested that glycosylation may play a regulatory role in transcription. *O*-linked glycosylated proteins are also phosphoproteins and the turnover of glycosylation is often associated with changes in enzymatic activity (Hart et al., 1989; Haltiwanger et al., 1992; Dahmus, 1994). We have not investigated whether the IIi subunit is glycosylated. It is entirely possible that glycosylation occurs on IIi since phosphorylation and glycosylation occur at multiple sites on the CTD and may be mutually exclusive (Dahmus, 1994). The physiological significance of RNAP II large subunit glycosylation remains to be determined. It would be of interest to examine whether the glycosylation state of the CTD is modified following HSV-1 infection.

2. RNAP II general transcription factors appear unmodified following HSV-1 infection

It is possible that HSV-1 may modify host RNAP II transcription by targeting and modifying the abundance or activities of other RNAP II GTFs or CTD kinases. However, we find no major changes in the phosphorylation state or protein abundance of several RNAP II GTFs and CTD kinases after HSV-1 infection. TBP (Chapter 6), the p56 subunit of TFIIE (Chapter 6), the RAP74 subunit of TFIIF (Chapter 6), the MAT1 and p62 subunits of TFIIH (Kilvert, 1995, Unpublished data), and CDK8 (Kilvert, 1995, Unpublished data) are constant in protein levels and phosphorylation state (as measured by SDS-PAGE) up to 12 hr post-infection. These data suggest, but do not rule out, the possibility that the virus does not target these GTFs or CTD kinases during infection.

Of the GTFs that we assayed, only TFIIB and MO15 protein levels appear to decline slightly following WT HSV-1 infection (Chapter 6). TFIIB binds to TBP-DNA complexes and aids in the recruitment of RNAP II and TFIIF to PICs (Buratowski et al., 1989; Conaway and Conaway, 1993; Zawel and Reinberg, 1993; Zawel and Reinberg, 1995; Orphanides et al., 1996). It has been reported that TFIIB is a component of the RNAP II holoenzyme (Koleske and Young, 1995; Ossipow et al., 1995). In addition, TFIIB may play a role in RNAP II transcription start site selection. (Orphanides et al., 1996). It is possible that HSV-1 targets TFIIB by down-regulating its expression (either transcriptionally or post-transcriptionally) in order to alter transcription start sites, perhaps on viral genes. At present, there is little evidence to support this hypothesis. We have some preliminary evidence that indicates that identical transcription start sites are used in uninfected and HSV-1 infected cells, at least during transcription *in vitro*. We have not further investigated the significance of TFIIB depletion after infection.

TFIIH is a multisubunit GTF that contains CTD kinase activity (Roeder, 1996; Svejstrup et al., 1996). MO15 is the CTD kinase component of TFIIH; however, it also forms a tripartite complex with cyclin H and

MAT1 and acts as a cyclin-dependent kinase activating kinase (CAK) (Poon et al., 1993; Mäkelä et al., 1994; Tassan et al., 1994). We do not know whether the reductions in MO15 abundance after HSV-1 infection occur in the TFIIH or CAK populations of MO15. However, we do note that MO15 is recruited into nuclear viral replication compartments following infection, suggesting that at least a portion of MO15 is available for viral RNAP II transcription. It is intriguing that HSV-1 infection depletes MO15 abundance, as this modification may contribute to the aberrant phosphorylation of RNAP II. If MO15 is a CTD kinase that functions *in vivo*, declines in its protein abundance after infection may facilitate the modification of the CTD by some other kinase. We have not yet conducted assays to determine whether HSV-1 infection alters the CTD kinase activity of MO15 or TFIIH.

3. The CTD kinase DNA-PK is depleted following HSV-1 infection

Several hypotheses could explain the mechanism by which HSV-1 aberrantly modifies the phosphorylation state of the RNAP II large subunit. These include: a) virus-induced loss of normal cellular CTD kinase activities, b) stimulation of CTD phosphatase activities, c) sequestration of RNAP II from cellular CTD kinases, and/or d) substitution of viral CTD kinases for cellular CTD kinases. In an effort to detect whether cellular CTD kinases were modified or depleted following HSV-1 infection, we examined the putative CTD kinases MO15, CDK8 and DNA-PK. As noted above, MO15 and CDK8 are relatively unchanged in abundance or migration on SDS-PAGE gels following HSV-1 infection. However, there are rapid and dramatic changes in the abundance and activity of the catalytic subunit of DNA-PK.

The depletion of p350/DNA-PKcs protein and DNA-PK activity after HSV-1 infection is dependent on the presence of the ICP0 protein. (Chapter 7, Lees-Miller et al., 1996). As described in Chapter 1, ICP0 is a nuclear phosphoprotein that binds to DNA *in vitro* (Hay and Hay, 1980; Everett et al., 1991b) and to chromatin *in vivo* (Hay and Hay, 1980). The function of ICP0 is not well understood, but it appears to act as a regulatory protein. In transient transfection assays, ICP0 transactivates RNAP II promoters;

however, its transactivation activities do not require any specific *cis*-acting sequence elements, including the TATA box (Nabel et al., 1988; Chen and Silverstein, 1992). The mechanism by which ICP0 targets DNA-PKcs for degradation is unknown. ICP0 binds to HAUSP, a novel member of the ubiquitin-specific protease family (Everett et al., 1997). The ubiquitin-dependent proteolytic pathway is responsible for the selective protein turnover in eukaryotes (Alberts et al., 1989). It is possible that ICP0 modulates the activity of HAUSP, thereby influencing the stability of specific cellular proteins such as p350/DNA-PKcs. As ICP0 has DNA binding activity, it is also possible that ICP0 may displace DNA-PK from DNA. The p350/DNA-PKcs subunit interacts with Ku only when Ku is bound to DNA; therefore, dissociation of DNA-PK from DNA may expose the p350/DNA-PKcs subunit to rapid degradation by normal cellular proteases. Alternatively, ICP0 could directly contribute to the loss of p350/DNA-PKcs by a specific proteolysis mechanism.

The depletion of DNA-PK activity does not appear to be solely responsible for RNAP II phosphorylation changes that occur following HSV-1 infection. The large subunit of RNAP II is efficiently modified after infection with both WT HSV-1 and ICP0 mutant viruses and therefore in the presence and absence of normal levels of p350/DNA-PKcs protein and DNA-PK activity. In addition, shutoff of host RNAP II transcription occurs after infection with both WT and ICP0 mutant viruses (Spencer et al., 1997). Therefore, host transcription shutoff is unaffected by the levels of p350/DNA-PK protein and DNA-PK activity. When cultured cells are infected with ICP0 mutant viruses (at an MOI of > 10 PFU/ml), virus replication and gene expression are normal, also suggesting that DNA-PK activity may not be needed for the viral life cycle (Sacks and Schaffer, 1987; Everett et al., 1991b) .

Despite these data suggesting that the presence of ICP0 and the depletion of DNA-PK depletion are incidental to normal viral gene expression and replication, it is still possible that ICP0 and DNA-PK play active roles in HSV-1 infection. Although ICP0 has few effects on viral growth and gene expression when ICP0 mutants infect cultured cells at high MOIs (> 10 PFU/ml), it appears to contribute to growth and DE and L gene expression when infections are performed at low MOIs (< 1 PFU/ml)

(Sacks and Schaffer, 1987; Everett et al., 1991b). In addition, ICP0 is required for the activation of latent virus in neurons (Leib et al, 1989; Cai et al., 1983). Therefore, under more natural situations (low MOI and latency), ICP0 plays an important role in the viral life cycle. It has been suggested that during ICP0 mutant infections performed at high MOIs, the abundant levels of VP16 introduced during the infection overcome the requirement for ICP0. VP16 and ICP0 may perform complementary roles in viral gene activation (Everett et al., 1991b; Cai and Schaffer, 1992). It is possible that DNA-PK may be inhibitory to gene expression during low MOI infections or reactivation from latency. There is evidence that DNA-PK represses RNAP II and RNAP I transcription (Kuhn et al., 1995; Labhart, 1995; Giffin et al., 1996; Giffin et al., 1997). It appears that glucocorticoid induced transcription of the mouse mammary tumor virus (MMTV) promoter is repressed by DNA-PK, perhaps by sequence specific binding of DNA-PK to a negative regulatory element and DNA-PK phosphorylation of the glucocorticoid receptor and OCT-1 (Giffin et al., 1996; Giffin et al., 1997). It is possible that DNA-PK represses RNAP II transcription of viral genes when viral DNA enters the nucleus, perhaps by binding to the viral DNA and then phosphorylating and inactivating transcription factors such as OCT-1. This inhibition may then be removed by virus-induced (via ICP0) depletion of p350/DNA-PK protein and DNA-PK activity. Similarly, DNA-PK may bind to latent viral DNA, and may need to be removed by ICP0 to allow activation of IE gene transcription during reactivation from latency.

Recently, DNA-PKcs and Ku have been identified as members of a human RNAP II holoenzyme (Maldonado et al., 1996). The significance of this association for RNAP II transcription and DNA repair has not yet been reported. Preliminary data from our lab indicates that DNA-PKcs remains associated with RNAP II following HSV-1 infection, although the levels of p350/DNA-PKcs are reduced from those in uninfected cells (Spencer, Unpublished data, 1997). These data suggest that virus-induced depletion of DNA-PKcs may not significantly affect the holoenzyme functions of DNA-PK. Therefore, DNA-PK depletion may affect other post-infection functions unrelated to RNAP II transcription. It is possible that depletion of p350/DNA-PKcs protein and DNA-PK activity after HSV-1 infection would affect DNA double-strand break repair (Anderson, 1994; Jeggo et al., 1995; Roth et al., 1995). By altering repair and recombination mechanisms, viral DNA replication or packaging may be enhanced. However, to date, ICP0 has not been demonstrated to have viral DNA replication or packaging functions.

C. Relocalization of RNAP II general transcription machinery following HSV-1 infection

Nuclear processes such as transcription, DNA replication and pre-mRNA processing show distinct intranuclear organization (reviewed in Hoffman, 1993). For example, topoisomerase II, transcription proteins and pre-mRNA processing components specifically localize to intranuclear foci (Hozak et al., 1993; Swedlow et al., 1993; Xing et al., 1993).

During HSV-1 infection, viral DNA synthesis occurs in discrete intranuclear sites termed DNA replication compartments. These compartments contain aggregates of the viral replication machinery and increase in number and size as infection proceeds (Quinlan et al., 1984; Randall and Dinwoodie, 1986; deBruyn Kops and Knipe, 1988). Some HSV-1 regulatory proteins specifically localize to viral DNA replication compartments. These include ICP4 (Randall and Dinwoodie, 1986; Knipe et al., 1987), ICP8 (Quinlan et al., 1984), and ICP22 (Chapter 9). Not all viral regulatory proteins are preferentially localized to DNA replication compartments following HSV-1 infection. ICP27 remains dispersed throughout the nucleus (Chapter 3; Knipe et al., 1987). ICP0 is also dispersed throughout the nucleus, with some preferential localization to replication compartments (Knipe et al., 1987).

RNAP II is localized to viral DNA replication compartments within 5 hr post-infection. The sequestration of RNAP II into DNA replication compartments occurs concurrently with RNAP II modifications during WT virus infection (Chapter 3). However, modifications to the large subunit of RNAP II occur efficiently in the absence of RNAP II recruitment into viral replication compartments (i.e. after infection with ICP4 and ICP8 mutant viruses, which do not replicate viral DNA or form DNA replication compartments) (Chapter 4). Conversely, recruitment of RNAP II into DNA replication compartments is not dependent upon RNAP II large subunit modification to IIi. RNAP II localizes to DNA replication compartments during infection with ICP22 or UL13 mutant viruses, which do not efficiently modify the RNAP II large subunit to IIi (Chapter 4 and 5). Although recruitment into viral replication compartments appears to be unrelated to IIi formation, we cannot rule out a requirement for loss of IIo in RNAP II localization.

The large subunit of RNAP II has been observed to localize to distinct nuclear foci, which are sites of DNA replication and transcription, in adenovirus infected cells (Jiménez-García and Spector, 1993), suggesting that recruitment of the host's transcription machinery may be a general mechanism employed by nuclear DNA viruses to facilitate preferential transcription of their genomes.

In addition to RNAP II, TBP (Chapter 6) and the MO15 subunit of TFIIH (Kilvert, 1995, Unpublished data) localize to DNA replication compartments following HSV-1 infection. There is also some evidence that the p56 subunit of TFIIE, the RAP74 subunit of TFIIF and the MAT1 subunit of TFIIH localize into these nuclear viral compartments (Kilvert, 1995, Unpublished data). These data suggest that most, if not all, components of the RNAP II holoenzyme may be recruited by the virus into DNA replication compartments. Preliminary data from our lab supports this idea, as the association of several GTFs with the holoenzyme appears unchanged following HSV-1 infection (Spencer, 1997, Unpublished data).

The relocation of RNAP II and GTFs to DNA replication compartments may be an active or a passive process. It is possible that viral regulatory proteins interact with members of the basal transcription machinery and directly increase their affinity for the viral genome. Alternatively, the RNAP II basal transcription machinery could simply be titrated onto transcribed viral genes following the shutoff of host transcription. Although we have not directly examined this question, our recent finding that ICP4 and ICP27 interact with the RNAP II holoenzyme is consistent with an active recruitment mechanism, which may be facilitated by the IE proteins ICP4 and ICP27 (Spencer, 1997, Unpublished data).

D. ICP22 AND UL13 are necessary for formation of IIi and for efficient DE and L gene transcription

1. ICP22 is required for formation of IIi and for normal patterns of viral gene transcription

We find that expression of the viral IE protein ICP22 is required to obtain efficient modification of the large subunit of RNAP II to the IIi form (Chapter 4). In addition, ICP22 is necessary for normal DE and L gene transcription in certain cell types (Chapter 4; Rice et al., 1995). Although ICP22 mutants (both 22/*n*199 and *d*22) fail to induce WT levels of IIi as measured by Western blot analyses, we observe subtle differences in IIi formation between Vero and HEL 299 cells. Low levels of IIi are detectable in ICP22 mutant infected Vero cells at late times of infection, whereas IIi remains undetectable in HEL 299 cells even at late times post-infection (Chapter 4).

ICP22 mutant viruses show cell type dependent growth phenotypes: they grow well in Vero cells, but poorly in HEL 299 cells (Astor et al., Unpublished data; Sears et al., 1985; Poffenberger et al., 1993). In addition, ICP22 mutants exhibit defects at the level of DE and L protein synthesis, particularly during restrictive infections such as those in HEL 299 cells (Astor et al., Unpublished data; Sears et al., 1985; Poffenberger et al., 1993). We find that ICP22 mutant viruses also show cell type dependent patterns of viral gene transcription: DE and L gene transcription is normal in ICP22 mutant infected Vero cells, but impaired in ICP22 mutant infected HEL 299 cells (Chapter 5; Rice et al., 1995). Therefore, defects at the level of protein accumulation may directly reflect transcription defects that occur in cell type specific ways. It has been suggested that ICP22 function may be complemented by host cell factors in permissive cells such as Vero cells (Purves et al., 1993; Sears et al., 1985).

The efficiency of IIi formation correlates with the efficiency of ICP22 mutant virus growth, DE/L gene transcription and DE/L protein accumulation in ICP22 mutant infected Vero and HEL 299 cells. It is tempting to speculate that ICP22 may affect the efficiency of viral gene transcription by altering RNAP II phosphorylation. However, it is also possible that ICP22 influences viral DE and L gene transcription which then indirectly affects RNAP II phosphorylation. We have yet to directly test these hypotheses by ICP22 expression experiments in uninfected cells.

Interestingly, hyperphosphorylated forms of the large subunit of RNAP II are the transcriptionally active forms after infection of both Vero cells and HEL 299 cells with ICP22 mutant viruses, as measured by photoaffinity labeling assays (Spencer et al., 1997). Therefore, even though hyperphosphorylated forms (IIi and IIo) are in very low abundance or undetectable as assayed by Western blotting (Chapter 4), these small amounts are transcriptionally active following infection with ICP22 mutant viruses. This suggests that transcription elongation, on both the viral and the cellular genes, is accompanied by hyperphosphorylation of the large subunit of RNAP II.

Although ICP22 is required for the modification of the large subunit of RNAP II to the IIi form, it is not required for the other modification that follows HSV-1 infection: the loss of IIo (Chapter 4). Similarly, although ICP22 is required for efficient DE and L gene transcription (at least in some cell types) (Chapter 5; Rice et al., 1995), it is not required for repression of host RNAP II transcription (Spencer et al., 1997). Both loss of IIo and repression of host transcription repression occur after infection with viruses mutant in each of the IE genes including ICP22, but not with UV-inactivated virions (Chapter 4). This suggests that several IE gene products can induce host transcription shutoff and loss of IIo. It also indicates that host transcription repression and loss of IIo do not depend on either the appearance of IIi or transcription of viral DE or L genes. It will be of interest to examine RNAP II modifications and host transcription repression after infection with viruses mutant in multiple IE genes.

2. UL13 is also required for formation of IIi and for normal patterns of viral gene transcription

We have found that the viral kinase UL13 is required for efficient modification of the large subunit of RNAP II to the IIi form (Chapter 5). This was in some ways unexpected, as DE and L gene expression are not required for RNAP II modification, and UL13 is a L gene product.

However, UL13 is also a virion component and is introduced into cells immediately after infection (Purves et al., 1986b; Frame et al., 1987; Zhang et al., 1990). Although virion components alone are not sufficient to modify RNAP II (Chapter 4), UL13 may work in combination with ICP22.

The phenotypes of UL13 mutants are very similar to those of ICP22 mutants. UL13 mutants display similar cell type dependencies for growth as ICP22 mutants (Purves et al., 1993; Overton et al., 1994): they grow well in Vero cells, but poorly in HEL cells. The efficiency of IIi formation in UL13 mutant virus infected Vero and HEL cells is similar to that in ICP22 mutant virus infected Vero and HEL cells. UL13, like ICP22, is required for normal patterns of viral DE and L gene transcription in HEL 299 (but not in Vero) cells (Chapter 5). Decreases in viral gene transcription correlate with decreases in protein abundance in restrictive infections (Purves et al., 1993). As in ICP22 mutant virus infections, IIo is depleted and host RNAP II transcription is repressed (Chapter 5). These data support the idea that ICP22 and UL13 act in the same pathway that leads to these phenotypes.

ICP22 is a phosphoprotein, whose normal patterns of phosphorylation depend upon the presence of UL13, which is a serine and threonine kinase (Overton et al., 1992; Purves and Roizman, 1992; Coulter et al., 1993; Purves et al., 1993). Although this suggests that ICP22 may be a substrate for UL13 phosphorylation, it has not yet been determined whether UL13's effects on ICP22 are direct or indirect.

How UL13, in conjunction with ICP22, leads to the modification of RNAP II phosphorylation is unknown. UL13 may indirectly induce IIi by phosphorylating ICP22, which in turn may trigger IIi formation. Alternatively, UL13 may directly function as an ICP22-dependent CTD kinase, in addition to being an ICP22 modifier. Another scenario is that UL13 leads to modification of RNAP II by phosphorylating another target, in an ICP22-dependent fashion. The other target may then induce RNAP II modifications. It would be interesting to directly test the CTD kinase activity of UL13 biochemically. Moreover, it would be of interest to test the functions of UL13 and ICP22 independently by expressing each in uninfected cells. As UL13 is virion associated and virion components alone are unable to modify the large subunit of RNAP II (Chapter 4), we do not expect that expressing UL13 alone will induce RNAP II modifications.

Unfortunately, to date we have been unable to express ICP22 from an inducible tet-VP16 construct stably transfected into HeLa cells. As explained in Chapter 9, we suspect that leaky expression of ICP22 from these constructs is sufficient to kill transfected cells. ICP22 has been reported to be toxic when expressed by transient transfection assays (Johnson et al., 1994).

E. ICP22 and ICP4 interactions

We find that F-ICP22 and ICP4 coimmunoprecipitate from nuclear and cellular extracts, and that the N terminus of ICP4 may mediate this interaction (Chapter 10). Interactions between IE proteins and between cellular and IE proteins have been reported (Smith et al., 1993; Yao and Schaffer, 1994; Carrozza and DeLuca, 1996; Leopardi and Roizman, 1996; Leopardi et al., 1997; Panagiotidis et al., 1997). Although these interactions are observed mainly *in vitro*, they may be significant *in vivo* and may mediate in part the regulatory functions of the IE proteins.

ICP4 interacts with RNAP II transcription factors, including TBP, TFIIB, and $TAF_{II}250$ (Smith et al., 1993; Carrozza and DeLuca, 1996). TBP, TFIIB and ICP4 form a tripartite complex on DNA, and the affinities of TBP and ICP4 for their respective binding sites is enhanced within this complex (Smith et al., 1993). In addition, ICP4 directly binds TFIID, through interactions with $TAF_{II}250$ (Carrozza and DeLuca, 1996). These data suggest that ICP4 may participate in PIC formation on viral promoters.

Both ICP4 and ICP22 interact with Epstein-Barr virus small RNAs (EBER)-associated protein (EAP) (Leopardi and Roizman, 1996; Leopardi et al., 1997), a host nucleolar protein that associates with ribosomes and binds to RNAs of unknown function in Epstein-Barr virus infected cells (Toczyski and Steitz, 1991; Toczyski and Steitz, 1993; Toczyski et al., 1994). ICP4 and EAP interactions decrease ICP4 binding to its cognate DNA sequence (Leopardi and Roizman, 1996), but the functional significance of ICP22 and EAP interactions is still a mystery. EAP has no known transcriptional activity and may merely serve as a bridger molecule for interactions between ICP4 and ICP22 with each other or with other cellular or viral proteins (Leopardi et al., 1997).

ICP4 also interacts with the IE proteins ICP0 and ICP27 (Yao and Schaffer, 1994; Mullen et al., 1995; Panagiotidis et al., 1997). Interactions between these proteins may modulate their respective functions. ICP0, ICP4 and ICP27 appear to function jointly in HSV-1 gene regulation. It is possible that ICP4 and ICP0 interactions stabilize ICP0 and ICP4 association with DNA, resulting in the synergistic gene activation observed in transient transfections assays with these two proteins (Yao and Schaffer, 1994). ICP27 inhibits or augments the combined stimulatory activities of ICP0 and ICP4 on DE and L gene promoters (Everett, 1986; Gelman and Silverstein, 1987; Su and Knipe, 1987; Rice and Knipe, 1988; Sekulovich et al., 1988; Rice et al., 1989). ICP27 may accomplish this by its interactions with ICP4. In sum, these data suggest that the IE regulatory proteins ICP4, ICP0, and ICP27 may form multiprotein complexes and may enact some of their functions within these complexes.

The function of ICP22 interactions with other IE proteins such as ICP4 is unknown. It is possible that ICP22 and ICP4 interactions serve to enhance ICP4 binding to DNA, which ultimately may improve ICP4's transcription stimulatory activity. There is evidence in the literature to support this hypothesis. The equine herpesvirus (EHV) homologue of HSV-1 ICP22 (EICP22) improves the *in vitro* DNA binding activity of the EHV homologue of HSV-1 ICP4 (EICP4) (Kim et al., 1997b). Although no interactions between ICP22 and TBP, $TAF_{II}250$, or TFIIB have been observed, ICP22 may modulate PIC assembly on viral promoters by its interactions with ICP4. ICP22 may also augment the associations of ICP4 with ICP27 and/or ICP0, thereby enhancing their combined effects. As ICP22 is required for efficient virus growth and gene expression only in some cell types, it is possible that any ICP22 enhancement of ICP4 function may be complemented with certain cellular factors. Preliminary data from our lab show that the IE proteins ICP4 and ICP27 interact with components of the RNAP II holoenzyme (Spencer, 1997, Unpublished data). Both IE proteins coimmunoprecipitate from nuclear extracts with antibodies against the RNAP II large subunit and the MO15 subunit of TFIIH. It is possible that the interactions we observe between ICP22 with ICP4 are part of more extensive interactions that involve several viral IE proteins and the RNAP II holoenzyme. The association of IE proteins with the RNAP II holoenzyme may alter holoenzyme biochemical activities, such as interactions with transcription activators, CTD phosphorylation and chromatin remodeling (refer to section G).

In an ICP4 (*d*120) mutant infection, IIi is efficiently induced (Chapter 4), suggesting that ICP22's interactions with ICP4 may not be important for RNAP II modifications. However, *d*120 encodes a truncated (30 kDa) ICP4 protein, consisting of the first 171 amino acids of the 1298 amino acid protein (Chapter 10; DeLuca et al., 1985). The truncated ICP4 protein is capable of interacting with F-ICP22 (Chapter 10). Therefore, it is still possible that the interaction between ICP22 and ICP4 may be important for ICP22 function.

Interestingly, the first 171 amino acids of ICP4 are necessary for ICP4's transactivation function (DeLuca and Schaffer, 1988; Shepard et al., 1989; Xiao et al., 1997). Therefore, it is possible that interactions between ICP22 and ICP4 may modulate ICP4's gene activation activity. It would be of interest to re-examine RNAP II modifications with an ICP4 mutant containing a complete deletion of the ICP4 gene.

Although the first 171 amino acids of ICP4 appear to interact with ICP22, they are not necessary for ICP4's interactions with other IE proteins. ICP0 interacts with ICP4 at ICP4's carboxy terminus (amino acids 1063 to 1298) (Yao and Schaffer, 1994). ICP27 appears to interact with ICP4 at several regions. The ICP4 regions important for ICP27 interaction include amino acids 245-523, 573-686, and 875-1298 (Panagiotidis et al., 1997).

The ability of ICP4 to form complexes with TBP, TFIIB and DNA maps between amino acids 142-274 (Smith et al., 1993), a region that partially overlaps with the region that interacts with F-ICP22. The N terminus of ICP4, between amino acids 138-262, is required for interactions with EAP (Leopardi and Roizman, 1996). These residues include a stretch of serines that are thought to be involved in protein interactions and are adjacent to the ICP4 DNA binding domain (DeLuca and Schaffer, 1988; Shepard et al., 1989). This serine-rich region of ICP4 is homologous to a region of a cellular protein (p15) that acts as a transcription mediator and interacts with TBP (Kretzschmar et al., 1994). The EAP interaction region of ICP4 partially overlaps with the first 171 amino acids which may be involved in F-ICP22 interaction. It is currently unknown whether F-ICP22 and ICP4 interactions are independent of EAP. The coimmunoprecipitation of F-ICP22 and ICP4 may be direct or facilitated by EAP.

Interactions between F-ICP22 and other proteins *in vivo* may not by detected under our stringent immunoprecipitation methods. Using these methods, we did not detect interactions between F-ICP22 and ICP0, ICP27, TBP, or the RNAP II large subunit (Chapter 10). It will be of interest to examine ICP22 interactions with RNAP II holoenzyme components and viral IE proteins using the coimmunoprecipitation methods employed for RNAP II holoenzyme studies (Ossipow et al., 1995; Chao et al., 1996; Maldonado et al., 1996).

F. Modifications to the RNAP II large subunit following infection with other human DNA viruses

The Herpesviridae family consists of three subfamilies: α-, β-, and γ-herpesviruses (Figure 1.1). The human α-herpesviruses include HSV-1, HSV-2, and varicella-zoster virus (VZV). Examples of the β- and γ-herpesviruses include the human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV). We find that the RNAP II large subunit is modified after infection with HSV-1, HSV-2 and VZV. RNAP II modifications appear with the same efficiency and kinetics in HSV-1 and HSV-2 infected cells. However, in VZV infected human melanoma (MeWos) cells, RNAP II modifications are more subtle, with less IIi present at late times post-infection. The degree of RNAP II modification following VZV infection does not appear to depend on the degree of cytopathic effect (cpe). Although we have not characterized the IIi subunits induced after VZV infection, we suspect that the large subunit of RNAP II is altered by phosphorylation, as in HSV-1 infected cells (Chapter 8).

The observation that RNAP II modifications occur after infection with other α-herpesviruses such as HSV-2 and VZV is not surprising, given the similarities among the α-herpesviruses. As in HSV-1 infection, cellular transcription is repressed while viral transcription is activated following HSV-2 and VZV infection. The HSV-2 and VZV genome is transcribed and replicated in the host's nucleus and these viruses utilize the cellular RNAP II transcription machinery to express its own genes. In addition, the expression of viral genes is regulated at the transcription level in a tightly controlled temporal cascade (Murphy and Kingsbury, 1990).

HSV-1 and HSV-2 show extensive similarities in their nucleotide and protein sequences. The HSV-1 and HSV-1 genomes contain 67% and 69% G+C content respectively (Roizman et al., 1981). The thymidine kinase, glycoprotein C, alkaline exonuclease and ribonucleotide reductase amino acid sequences are 86%, 82%, 91%, 93% homologous in HSV-1 and HSV-2 (Draper et al., 1986). A comparison of the nucleotide sequence and the amino acid sequence of ICP8 between HSV-1 and HSV-2 show 89.9% and 97.2% homology respectively (Toh et al., 1993). The level of amino acid sequence conservation between HSV-1 and HSV-2 ICP22 proteins is over 50% near the N-termini (Kilvert, 1995, Unpublished data; Whitton and Clements, 1984). The conservation between HSV-1 and HSV-2's UL13 proteins has not been determined. However, HSV-1 UL13 is similar to HSV-2 UL13 in molecular mass, and antibodies raised against HSV-1 UL13 efficiently recognize HSV-2 UL13 (Overton et al., 1992). This suggests that some homology exists between HSV-1 and HSV-2's UL13 proteins.

VZV is not as closely related to HSV-1 as HSV-2 is, in terms of DNA or protein sequence homology. Although each VZV gene has an HSV-1 counterpart, six HSV-1 genes do not have a VZV homologue (Davison and McGeoch, 1986). The VZV genome contains 46% G+C (Roizman et al., 1981). VZV shares some DNA sequence homology with HSV-1, especially in the unique short regions and adjacent repeats. As demonstrated in previous studies, VZV complements certain HSV-1 temperature sensitive mutants. The VZV homologues of the HSV-1 IE genes ICP4 and ICP27 are able to complement HSV-1 temperature sensitive mutants and allow them to replicate more efficiently (Felser et al., 1987). We have not characterized the VZV gene products that are required for RNAP II modification. The VZV homologues of HSV-1's ICP22 and UL13 may be involved in IIi induction following VZV infection. It would be of interest to determine if the subtle modifications in the RNAP II large subunit are necessary for efficient VZV gene transcription.

Indirect immunofluorescence studies show that RNAP II is localized to distinct nuclear foci after VZV infection of MeWos cells (Ruyechan and

Stevenson, 1995, Unpublished data). These nuclear foci contain the VZV ORF 29 protein, the VZV homologue of HSV-1's DE gene product ICP8 (Kinchington et al., 1988). The ORF 29 protein is the major DNA binding protein of VZV (Kinchington et al., 1988). These localization results resemble our findings demonstrating RNAP II and ICP8 colocalization into DNA replication compartments following HSV-1 infection (Chapter 3). However, in a VZV infection, the recruitment of RNAP II into the nuclear foci containing VZV ORF 29 protein is not as complete as observed in an HSV-1 infection. The partial recruitment of RNAP II into discrete nuclear foci may play a role in the selective transcription of VZV genes.

Infection of human cells with the β-herpesviruses, HCMV and human herpesvirus type 6 (HHV-6), result in no detectable modifications to the large subunit of RNAP II. These results are not entirely surprising. Although the transcription of HCMV and HHV-6 genes is similar to HSV-1 in that gene expression is controlled in a temporal cascade and is dependent on the host's RNAP II transcription machinery, β-herpesviruses differ from HSV-1 in terms of DNA and amino acid sequences. HCMV shows limited DNA sequence homology with other human herpesviruses and displays at least 50% more sequence complexity than HSV (duplications, sequence divergence) (Stinski, 1990). HHV-6 shares little DNA and amino acid sequence homology with HSV but is most related to HCMV (Efstathious et al., 1988; Lawrence et al., 1990). There is a 66% nucleotide sequence identity between HHV-6 and HCMV in certain ORF regions (Lawrence, 1990).

Infection of human cells with vaccinia virus (VV), an even more distantly-related virus to HSV-1, results in no detectable modifications to the large subunit of RNAP II (Chapter 8). These results are not unanticipated since VV is a cytoplasmic DNA virus that utilizes its own RNAP to transcribe viral genes. Therefore, VV may not require modifications to cellular RNAP II in order to actively express its genes. Interestingly, the RNAP II large subunit translocates from the nucleus to the cytoplasm after VV infection, associates with virosomes and is packaged into virion particles (Chapter 8; Morrison and Moyer, 1986; Wilton and Dales, 1986). However, modifications to RNAP II appear not to accompany these changes to RNAP II localization.

G. Models for HSV-1 induced host transcription repression and viral transcription activation

In this study, we have addressed the question of how RNA polymerase II transcription is activated on some genes and repressed on others, by analyzing the changes that occur to the host basal transcription machinery following infection with HSV-1. Results of this study suggest that virus-induced modifications to components of the RNAP II transcription apparatus may contribute to the shift in transcription from cellular to viral genomes.

Any model to explain how RNAP II transcription is activated on viral genes and repressed on cellular genes must take into account the evidence that suggests the involvement of sequence-independent mechanisms. As described previously, viral genes and their promoters are regulated differently when present in the virus than when inserted into the cellular genome (Silver and Roizman, 1985). Conversely, cellular genes and their promoters become regulated as viral genes and promoters when inserted into the viral genome (Smiley et al, 1987; Panning and Smiley, 1989; Smibert and Smiley, 1990; Smiley et al., 1991). Two general models can be invoked: the active and the passive models. My reasons for favoring the active model are explained below.

1. Passive model: HSV-1 represses host gene transcription and activates viral DE and L gene transcription passively or indirectly

In the passive model, the viral genome is highly accessible for association with RNAP II transcription factors. This may be facilitated by HSV-1's nonnucleosomal chromatin configuration or by binding of viral proteins such as ICP4, ICP0, or ICP8 to viral templates. In this way, viral gene promoters are more readily accessible for transcription, and over a few hours post-infection, TFIID and other GTFs are titrated to the viral genome. Eventually, cellular genes are depleted of RNAP II and GTFs. In this model, the IE genes are transcriptionally activated by VP16 and associated cellular factors and by transcription initiation mechanisms that are similar for both cellular and viral genes. Once the IE proteins have accumulated, the viral genome becomes remodeled into the fully accessible state. As viral DNA replication begins, more nonnucleosomal viral genomes become available for RNAP II and GTF association, and the transition to viral gene transcription is complete. In the passive model, the disappearance of RNAP IIO and the appearance of RNAP II$_I$ are irrelevant to the transcription switch from cellular to viral transcription and are merely benign symptoms. RNAP II$_I$ may be the elongating form because host kinases or phosphatases have been altered, depleted or separated from the RNAP II transcription machinery following sequestration of RNAP II into DNA replication compartments. The virus may not require the formation of IIi, but may tolerate it because of the nonnucleosomal configuration of the viral genome.

Although we cannot rule out the passive model, several pieces of evidence may oppose it. Firstly, the shutoff of host transcription, the loss of RNAP IIO and the appearance of RNAP II$_I$ are efficient in an ICP4 mutant virus infection (Chapter 4; Smiley et al., 1987; Spencer et al., 1997). In ICP4 mutant infected cells, DE and L genes are not transcribed and DNA replication does not occur; only IE genes are transcribed and translated. In an ICP4 mutant virus infection, the number of available IE promoters that could titrate TFIID and RNAP II basal factors would be approximately 600 per cell (MOI of 10, particle to PFU ratio of 10, and 6 IE genes per genome, i.e. 10x10x6=600). Although the number of transcriptionally active RNAP II genes per cell is unknown, it has been estimated that 10,000 to 20,000 genes are actively transcribed in a normal cell (Alberts et al., 1994). Therefore, a minimum of about 10,000 TFIID molecules would be associated with cellular RNAP II genes. A viral titration of 600 of these 10,000 TFIID molecules would not severely compromise the activity of cellular RNAP II promoters. It is possible that DE and L promoters also titrate TFIID and RNAP II in an ICP4 mutant infection, even though they are not transcriptionally active. If this was the case, the 75 HSV-1 genes would titrate out 7,500 TFIID and RNAP II basal factors per cell, which may cause the severe cellular repression observed after infection. A minimum of 2,500 TFIID and RNAP II basal factors would only be available for cellular transcription. Secondly, infection of cells with UV-inactivated virions has no significant effect on host transcription shutoff or RNAP IIO abundance (Chapter 4; Spencer et al., 1997). UV-inactivated virions contain active VP16 and intact viral DNA. The UV crosslinking of viral DNA prevents transcription elongation, but may not impair the association of RNAP II and GTFs with the IE and/or DE and L promoters. Therefore, the passive titration of RNAP II transcription factors to the viral gene promoters is not sufficient to trigger host transcription shutoff and RNAP II modifications. Expression of the IE genes is necessary. Thirdly, in our cycloheximide inhibition experiments, HSV-1 infections in the presence of cycloheximide allow transcription of the IE genes and association of RNAP II with DE and L gene promoters (if this occurs early in infection). DE and L translation is prevented (Chapter 4). In the presence of cycloheximide, RNAP II is not modified. However, only after cycloheximide washout and resumption of translation is RNAP II modified to the II_I form. Although these experiments examined only RNAP II modifications, and not host transcription shutoff, these data suggest that it is the presence of IE proteins, and not merely the activation of their gene promoters, that is necessary for transcription changes.

2. Active models: HSV-1 represses host gene transcription and activates viral DE and L gene transcription actively or directly

Our data, and that of others, rules out a role for viral DNA replication, expression of DE or L genes, or the sole presence of virion components in bringing about RNAP II and transcription changes. The data indicate that IE proteins must be present. In addition, our results suggest that the presence of IE proteins may be sufficient (perhaps in conjunction with virion components) for RNAP II modifications and transcription changes. Of the IE proteins, ICP22 (along with UL13) is necessary for efficient production of RNAP II_I. Furthermore, ICP22 and UL13 are required for efficient DE and L gene transcription, at least in some cell types. Also, at least two of ICP4, ICP0, ICP27 and ICP22 are redundant in their ability to trigger host shutoff (Spencer et al., 1997) and the loss of RNAP IIO (Chapter 4). Two variations of the active model are the repressor model and the basal transcription machinery model.

a. The repressor model

In the repressor model, two or more IE proteins repress host transcription directly, by acting as genome-specific transcription repressors. Once cellular genes are transcriptionally repressed, RNAP II and GTFs become available for transcription of the viral genome.

The mechanisms by which IE proteins could directly repress transcription are not known. ICP4 represses transcription of viral genes that contain an ICP4 binding site within their core promoters. Both ICP4 and ICP0 can bind DNA in nonspecific ways, which may contribute to disruption of host transcription factors in or near RNAP II promoters. Recently, ICP27 has been shown to contribute to full transcription shutoff of the host genome (Spencer et al., 1997). In keeping with the hypothesis that IE proteins may interfere with a fundamental host function, the expression of ICP0, ICP27 or ICP22 in cell lines is cytotoxic (Johnson et al., 1994). In addition, cells survive after infection only if all three of ICP4, ICP27 and ICP22 are mutated (Wu et al., 1996).

However, most evidence indicates that the IE proteins act as gene expression activators. ICP4 acts as a potent transcription activator in transient transfection assays (Everett, 1984; DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985). If ICP4 acts as a host transcription repressor, it does not do so universally, as it appears to activate some cellular genes (Kanangat et al., 1966; Kemp and Latchman, 1988a; Kemp and Latchman, 1988b; Preston, 1990; Cheung et al., 1997). Although the *in vivo* role of ICP0 in HSV-1 replication is still unclear, transient transfection assays demonstrate that ICP0 is a transactivator and not a repressor. ICP27's role may also be as a transcriptional activator, at least for specific DE genes (Samaniego et al., 1995; McGregor et al., 1996; Uprichard and Knipe, 1996).

Although the direct repressor model is possibly correct, to date no evidence exists to strongly support it.

b. The basal transcription machinery model

In the basal transcription machinery model, two or more IE proteins repress host transcription and modify RNAP II by directly interacting with the basal RNAP II transcription machinery. These interactions result in the relocation of the basal transcription machinery from cellular to viral genomes. As a result, there may be preferential transcription initiation on viral promoters and a loss of the fully phosphorylated form of the RNAP II large subunit. There are two aspects of this model, which may operate simultaneously or separately: disruption of TFIID associations and disruption of RNAP II holoenzyme composition.

i. Disruption and recruitment of TFIID

In the stepwise and some of the holoenzyme models of transcription initiation, TFIID binding to the TATA box is the nucleation step that sets up a promoter for PIC assembly. It is possible that two or more IE proteins could actively recruit TBP and TAFs to viral DE and L genes. This may occur through direct interactions between IE proteins and GTFs, which may result in the active recruitment of GTFs to nuclear sites of viral DNA transcription, via affinity of IE proteins for viral DNA or promoters. The disruption of TFIID binding to cellular gene promoters would be an active process, as TFIID binding is thought to be relatively stable through multiple rounds of transcription (Hernandez, 1993, Orphanides et al., 1996). Once TFIID is recruited to viral gene promoters, another function, conferred by the presence of ICP4, ICP27 and ICP22 (in some cell types), results in the transcription activation of DE and L genes.

In support of this model, ICP4 is known to interact with $TAF_{II}250$, and forms tripartite complexes on DNA with TFIIB and TBP (Smith et al., 1993; Carrozza and DeLuca, 1996). We have observed the recruitment of TBP into viral replication compartments early in infection (Chapter 6). In addition, all viral genes have TATA box sequences, and their expression is dependent upon the presence of these TATA sequences (Roizman and Sears, 1990).

Despite the data linking ICP4 and TBP/TFIID, repression of cellular transcription and modifications to RNAP II occur in the absence of ICP4, indicating that these functions may be performed by other IE proteins. We must note, however, that examination of host transcription shutoff and RNAP II modifications in ICP4 mutant virus infections were performed with the mutant virus, *d*120, which synthesizes a stable 171 amino acid truncated protein (Chapter 10; DeLuca et al., 1985; DeLuca and Schaffer, 1988). Although *d*120 is a null mutant in terms of viral gene activation, the truncated ICP4 protein may have some activity with regards to host transcription repression and RNAP II modifications. It will be interesting to examine these phenomena after infection with a virus lacking the entire ICP4 open reading frame.

ii. Disruption and recruitment of the RNAP II holoenzyme

In this model, two or more IE proteins associate with the RNAP II holoenzyme, modifying its normal activities so that it becomes incompetent for transcription of cellular genes but remains competent for initiation and elongation of viral genes.

Holoenzyme components vary in different preparations, but holoenzymes in yeast and humans exhibit several activities. Holoenzymes are capable of basal transcription as all, or many, of the RNAP II GTFs are present in the holoenzyme. In some holoenzyme preparations, TBP and TAFs are also present (Koleske and Young, 1995; Ossipow et al., 1995). Holoenzymes respond to transcription activators and remodel chromatin. In addition, holoenzymes phosphorylate the CTD of the RNAP II large subunit. CTD phosphorylation activity may be attributed to MO15 (TFIIH), DNA-PK and SRB10/SRB11 (CDK8/cyclin C).

The SRB/SWI/SNF complex, which interacts with the CTD of the RNAP II large subunit, mediates the holoenzyme's response to transcriptional activators (Kim et al., 1994; Liao et al., 1995; Wilson et al., 1996) and provides the holoenzyme with chromatin remodeling (Imbalzano et al., 1994; Kwon et al., 1994; Peterson and Tamkun, 1995; Wilson et al., 1996) and CTD phosphorylating abilities (Liao et al., 1995; Chao et al., 1996).

In the RNAP II holoenzyme disruption model, several steps may occur:

1. Immediately after HSV-1 infection, VP16 and associated cellular factors activate IE genes at *cis*-acting elements upstream of each IE gene. The

normal cellular holoenzyme and TFIID are recruited to IE promoters by the same mechanisms that recruit them to cellular promoters.

2. After virus infection, the IE proteins accumulate and directly associate with the RNAP II holoenzyme. We suggest that ICP4, ICP27, ICP0 and ICP22 (along with UL13) may all interact with the holoenzyme as a complex.

3. The association of IE proteins with the RNAP II holoenzyme disrupts the mediator complex from the holoenzyme. This disruption has several effects: it reduces the holoenzyme's ability to respond to transcription activators, it inhibits the holoenzyme's capacity to remodel chromatin, and it alters the holoenzyme's ability to phosphorylate or dephosphorylate the CTD. The post-infection holoenzyme is hence rendered incompetent for initiation on cellular promoters that are embedded in cellular chromatin and in most cases require activators that are bound to proximal-promoter and enhancer elements. In addition, the defect in CTD phosphorylation may impair the release of RNAP II from mediator components or transcription activators at cellular promoters. RNAP II may also be rendered incompetent for transcription elongation through chromatin, if CTD hyperphosphorylation (to IIo) is necessary for this effect. The association of IE proteins with the holoenzyme may also help to physically relocate the holoenzyme, if one or more of the IE proteins (such as ICP4) have an affinity for viral DNA.

4. If TFIID is part of the RNAP II holoenzyme, it may lose its affinity for cellular promoters through the active relocation of the entire holoenzyme complex to the viral genome. If TFIID is separate from the holoenzyme, it may be relocated independently to viral genes (as described above), or alternatively, it may lose its ability to stably associate with cellular promoters in the absence of the chromatin remodeling functions of the RNAP II holoenzyme.

5. The modified holoenzyme may still initiate transcription on cellular promoters that are relatively free of nucleosomes and may not require

transcription activators. However, most cellular genes would cease to be actively transcribed by the modified holoenzyme. Viral DE and L genes, with their simple promoters and nucleosome-free state, would be fully accessible to the modified RNAP II holoenzyme. In addition, the reduced capacity of the holoenzyme to convert RNAP IIA to hyperphosphorylated IIO forms would be tolerated by viral genes, as RNAP II would no longer need to be released from the mediator complex or transcription activators at viral promoters. In addition, RNAP II would no longer require hyperphosphorylation in order to elongate, as viral genes are not compacted into nucleosomal structures. The small amount of hyperphosphorylation required for transcription elongation on viral genes could be provided by ICP22/UL13 kinase (or by cellular kinases in some cell types).

Although several IE proteins may disrupt the mediator complex from the RNAP II holoenzyme, each IE protein may also confer its individual traits to the holoenzyme:

1. ICP4's presence in the holoenzyme may tether the holoenzyme to viral DNA via its nonspecific and specific DNA binding abilities (Roizman and Sears, 1990), as well as to promoter regions via its ability to form complexes with TFIIB, TBP, and $TAF_{II}250$ on DNA (Smith et al., 1993; Carrozza and DeLuca, 1996). ICP4 may also aid TBP binding to DNA (Smith et al., 1993).

In ICP4 mutant infections, the absence of ICP4 would still allow for a disrupted holoenzyme (through the actions of ICP27, ICP0 and ICP22). The disrupted holoenzyme would be incompetent for cellular transcription, but specific transcription of DE and L genes would not occur, as the holoenzyme would not be recruited to viral promoters. Transcription by the altered holoenzyme may still continue on IE genes and on a few cellular genes that are packaged in a nonnucleosomal state.

2. ICP27's presence in the RNAP II holoenzyme may not only disrupt the mediator association with the holoenzyme, but ICP27 may also alter the holoenzyme's interactions with RNA splicing and polyadenylation/cleavage factors, which are also thought to be either part

of the holoenzyme (McCracken et al., 1997) or associate with the CTD (Yuryev et al., 1996; Du and Warren, 1997). In this way, the presence of ICP27 may inhibit host splicing or may alter aspects of host or virus polyadenylation.

It is not known whether the IIi form interacts with components of the splicing machinery. If IIi is incapable of interacting with the splicing factors, these factors may not be brought to the sites of pre-mRNA processing. As a result, HSV-1 infected cells may not be able to efficiently process cellular pre-mRNA. Previous studies have shown that during virus infection, host cell splicing is inhibited (Hardy and Sandri-Goldin, 1994). The absence of RNAP IIO during HSV-1 infection may not affect the production of mature viral mRNAs, because most viral genes lack intronic sequences.

In ICP27 mutant infections, the absence of ICP27 in the holoenzyme would still allow holoenzyme disruption (through the actions of ICP4, ICP22 and ICP0), and hence shutoff of host transcription and modifications to RNAP II phosphorylation would still occur. However, the post-transcriptional effects that accompany transcription (splicing and polyadenylation) may be more normal after infection with ICP27 mutant viruses.

3. ICP22/UL13's presence in the holoenzyme may both disrupt the mediator complex and may also allow efficient modification of the CTD during transcription on viral genes, particularly in situations (certain cell lines) where cellular CTD kinase activities are at low levels post-infection.

In ICP22 or UL13 mutant infections, the mediator complex would still be disrupted from the holoenzyme (via the presence of the other IE proteins), host transcription would be repressed and RNAP II CTD phosphorylation would be impaired. In some cell lines, residual cellular CTD kinase activity would be sufficient to confer some hyperphosphorylation to the large subunit during viral gene transcription. In cell lines with very low residual CTD kinase activity, less hyperphosphorylation of the CTD would be apparent, and if low enough, might impair transcription initiation or elongation on viral DE and L genes.

4. The presence of ICP0 in the holoenzyme may augment the activities of ICP4, such as tethering the holoenzyme to viral DNA or DE/L gene promoters. This might account for ICP0's ability to act synergistically with ICP4 in transactivation.

In infections in cultured cells with ICP0 mutants at high MOIs, little effect on gene expression would be detected, due to the presence of large amounts of VP16 and IE proteins such as ICP4 (Sacks and Schaffer, 1987; Everett et al., 1991b; Cai and Schaffer, 1992). ICP0's stimulatory effect on holoenzyme activation might only be critical when there are low amounts of other activators, such as VP16 or ICP4.

In this way, the association of IE proteins with the RNAP II holoenzyme (and TFIID, if it is not a part of the holoenzyme) may result in the observed shifts in transcription from cellular to viral genes. In addition, the association of IE proteins with the RNAP II holoenzyme may result in a change in the phosphorylation state of the CTD during transcription of the viral DE and L genes.

H. Future experiments

Modification of RNAP II and efficient viral transcription (at least in certain cell lines) are dependent on the viral proteins ICP22 and UL13. Our experiments suggest that ICP22 and UL13 work in combination to cause these two phenomena. Further characterization of the ICP22 and UL13 proteins may provide information on the functional domains in each of these proteins in terms of their respective roles in RNAP II phosphorylation, viral transcription and protein-protein interactions. Whether ICP22 and UL13 in the absence of virus infection are sufficient to create RNAP II_I is also unknown. It will be of interest to determine if UL13 is an ICP22 dependent CTD kinase or whether UL13 and ICP22 affect the activity of another cellular or viral CTD kinase in order to elicit RNAP II modifications and changes in viral transcription.

The characterization of RNAP II holoenzymes after HSV-1 infection will provide us with a greater understanding of how the shifts in transcription from cellular to viral genes occur. The effects of IE proteins on holoenzymes may be instrumental in disrupting cellular transcription and activating viral transcription, by altering the holoenzyme's ability to respond to transcriptional activators, properly phosphorylate the CTD, and/or remodel chromatin. Whether ICP4, ICP27 or other IE proteins disrupt or alter normal RNAP II holoenzyme activities remains to be determined.

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